

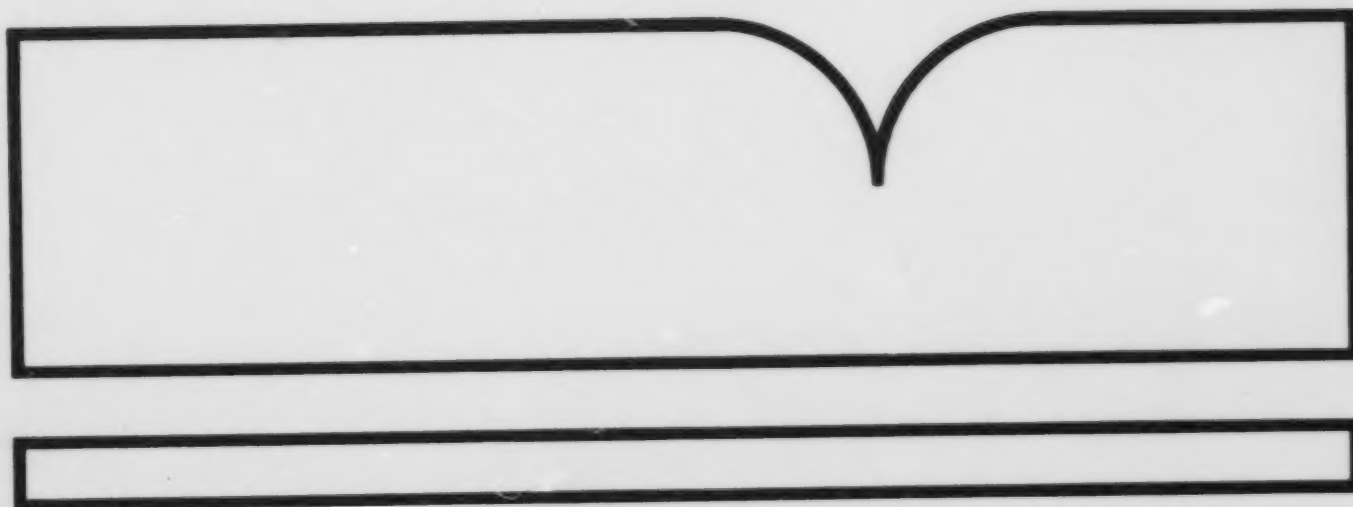
Study of Pathogenic Free-Living Amebas in
Fresh-Water Lakes in Virginia

Virginia Commonwealth Univ., Richmond

Prepared for

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STUDY OF PATHOGENIC FREE-LIVING
AMEBAS IN FRESH-WATER LAKES
IN VIRGINIA

by

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FOREWORD

The U.S. Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. Noxious air, foul water, and spoiled land are tragic testimony to the deterioration of our national environment. The complexity of that environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The primary mission of the Health Effects Research Laboratory in Cincinnati (HERL) is to provide a sound health effects data base in support of the regulatory activities of the EPA. To this end, HERL conducts a research program to identify, characterize, and quantitate harmful effects of pollutants that may result from exposure to chemical, physical, or biological agents found in the environment. In addition to the valuable health information generated by these activities, new research techniques and methods are being developed that contribute to a better understanding of human biochemical and physiological functions, and how these functions are altered by low-level insults.

This report describes and interprets the results of a three-year study of nine different fresh-water lakes located within a 27-mile radius in the Richmond, Virginia, area, a locale known to be endemic for fatal Primary Amebic Meningoencephalitis, a disease due to free-living amebas of the genus Naegleria. Included were determinations and comparisons of the protozoological, bacteriological, and physiochemical aspects of each lake, as well as concurrent climatological factors. In addition, the effect of thermal enrichment was considered. A better understanding of these factors may allow development of measures to reduce exposure.

R. J. Garner
R. J. Garner
Director
Health Effects Research Laboratory

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ABSTRACT

Pathogenic free-living amebas may produce a fatal infection of the central nervous system of man known as Primary Amebic Meningoencephalitis (PAM). In Richmond, Virginia, 17 such cases have occurred to date, more than in any other geographic location in the world. The major objectives of this study were to examine fresh-water lakes in the vicinity of Richmond, Virginia, for the presence of pathogenic free-living amebas, particularly Naegleria, and to gain some understanding of their prevalence and the conditions or factors which might affect their existence or concentrations.

Nine lakes, all within a 24-mile radius but with different limnological characteristics, were selected for biweekly sampling and study. Two of the lakes were previously shown to be the source of several human infections. Techniques for isolating and studying amebas from the environment were either modified from the methods of others or newly developed. Although ameboflagellates consistent with Naegleria were often isolated from all lakes, pathogenic ones were found infrequently. The major reason for this finding appeared to be the temperate climate and the short periods of time (days to weeks) that sustained elevations of water temperature ($>26^{\circ}\text{C}$) occurred, unlike the situation observed in the more tropical areas of Florida.

Soil samples proved to be more dependable sources than water from which amebas could be isolated, particularly those amebas growing at 44°C and those that were pathogenic. The techniques of soil sampling and testing were recommended as simple, reliable, and of potential usefulness to public health and environmental officials for screening for pathogenic Naegleria. Pathogenic amebas were isolated only from shallow bottom soil and its associated water (depth, six to ten inches), but not from deep water samples.

During biweekly attempts at isolating amebas, temperature, pH, dissolved oxygen, salinity, and conductivity of the water were measured. Of these, water temperature appeared to correlate best with the presence of amebas, and when it exceeded 30°C , pathogenic forms might be expected to be isolated. In addition, daily climatological data were assembled; and on several occasions each lake was studied extensively both chemically and biologically by the Environmental Studies Group at Virginia Polytechnic Institute and State University (VPISU). The most enriched lakes did not necessarily yield frequent or large numbers of pathogenic amebas.

The bacteriologic flora of the lakes studied was also examined. Although no quantitative (MPN) relationship of the presence of coliform bacteria to pathogenic amebas or ameboflagellates could be shown, qualitatively there may have been a relationship. Two types of bacteria frequently iso-

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lated from lake waters, Pseudomonas fluorescens and Serratia marcescens, appeared in the laboratory to be inhibitory to pathogenic Naegleria; and indeed in virtually every lake from which Ps. fluorescens was consistently isolated, no pathogenic Naegleria were found.

Of the lakes studied, the North Anna Reservoir or complex containing several coolant lagoons and canals for a nuclear power plant was the most intriguing; for the majority of pathogens and the most virulent organisms were isolated from this complex of lakes. Studies of this complex suggested that if natural fresh waters are artificially heated (thermal enrichment) for a sustained period of time, pathogenic Naegleria may be selected or concentrated, even in a cold or hostile climate, possibly serving as a source or nidus for contaminating other nearby lakes or streams. This study represented the first of its kind in which thermally enriched waters were studied before, during, and after thermal enrichment occurred; i.e., three sites at the North Anna Reservoir were studied biweekly for over a year before thermal enrichment was initiated and for over a year during and after which thermal enrichment occurred. Pathogenic Naegleria were isolated only with thermal enrichment, but not before or after.

This report was submitted in fulfillment of Grant No. R804014 from the U.S. Environmental Protection Agency and covered a period from 10/1/76 to 9/30/79. The work was completed as of 1/1/80.

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SECTION 1

INTRODUCTION

OBJECTIVES

The aims of this study were to examine a number of fresh-water lakes in the vicinity of Richmond, Virginia, in an attempt to isolate and study pathogenic free-living amebas (particularly of the genus Naegleria) and to acquire some understanding as to the prevalence of such organisms in this locale and the condition(s) or selective force(s) which might control their appearances.

BACKGROUND INFORMATION

For many years free-living amebas, although highly ubiquitous in nature, were virtually ignored by medical scientists. They were considered harmless and more of a biologic curiosity than any possible threat to health. Usually, such organisms were encountered in the bacteriology or virology laboratories as contaminants of plate or cell cultures; or they were selected by biologists or protozoologists as the subject of a doctoral thesis (1,2).

However, in 1958, as serendipity would have it, an observation by Culbertson et al. at the Eli Lilly Research Laboratories changed everyone's views entirely concerning the potential virulence of such organisms (3). In what initially was suspected as an unsuitable lot of vaccine containing live infective polio virus, a free-living ameba, recently assigned the appropriate name Acanthamoeba culbertsoni, was discovered. The ameba proved highly pathogenic for primates, as well as for a variety of other animals, producing a fatal meningoencephalitis (4). From these observations and subsequent ones, Culbertson et al. correctly surmised that natural infections in man and other animals due to these organisms might occur.

Shortly thereafter in 1965, almost simultaneously, there appeared a report from South Australia by Fowler and Carter (5) of four fatal cases of amebic meningitis recognized retrospectively through autopsy studies and a report from Florida by Butt (6) of the occurrence of several fatal human cases of what appeared to be the same disease. In both reports the responsible pathogens proved to be free-living amebas, subsequently identified as members of the genus Naegleria, which represented ameboflagellates different from but related to the Acanthamoeba that Culbertson had discovered. Thus, a new disease was recognized; and investigators became concerned as to whether or not it was a new entity or an old one that had been overlooked, an issue that still has not been completely resolved.

Nevertheless, subsequent to the observations of Fowler and Carter and of Butt, numerous reports began to appear in the medical literature testifying to the worldwide distribution of the disease and the virulence of the responsible pathogens (7). It was soon discovered that two genera of free-living amebas appeared to be responsible for producing fatal meningoencephalitis in man: Naegleria and Acanthamoeba. The former organism was almost invariably acquired from intimate contact with fresh water; frequently resulted in epidemics; and produced predictably an acute, fulminant, rapidly fatal disease characterized by hemorrhagic necrosis of the brain in an otherwise healthy individual, and entering the central nervous system via the nasal passage and olfactory nerve route. The latter organism differed, however, as it was not necessarily acquired from contact with fresh water (in fact the epidemiology remains completely obscure); appeared to occur sporadically; often produced a subacute or chronic (but likewise, fatal) disease, characterized by a granulomatous as well as a neutrophilic reaction; often occurred in an immunocompromised patient; and appeared to enter the central nervous system not only by the olfactory route but perhaps more frequently via hematogenous spread from other parts of the body (7). The disease produced by either of these organisms became known as "Primary Amebic Meningoencephalitis" or PAM to distinguish it from secondary spread of infection to the brain due to Entamoeba histolytica, a parasite, poorly tolerant of oxygen, which commonly produced a primary infection of the colon known as amebiasis.

To date PAM due to Naegleria has been reported from every continent except the Arctic and Antarctica: from England, Ireland, Belgium, Czechoslovakia, East Africa, India, Korea, Australia, New Zealand, Venezuela, and the states of Virginia, Florida, Georgia, North Carolina, Pennsylvania, New York, Arkansas, Texas, Louisiana, Arizona, and California. From a public health and environmental standpoint, the most disturbing features of the disease due to this organism were its frequent occurrence in epidemic proportions and its acquisition from fresh water, respectively; e.g., the common source epidemics and sporadic cases in Florida were traced to several fresh-water lakes in the Orlando area used for recreation; the epidemics in Virginia were traced to three fresh-water lakes within a five mile radius used by the public for swimming and diving; the outbreak in Czechoslovakia was traced to a heated swimming pool in Bavaria; similarly, the outbreak in Belgium was traced to a heated indoor pool; the cases in New Zealand and California to natural thermal pools; recent outbreaks in England to mineral baths; and most disturbing in Australia to contamination of the public potable water supply for the entire Spencer Gulf area. However, despite the apparent ubiquitous nature of the responsible organisms and the widespread distribution of outbreaks and sporadic cases, the disease appeared to be endemic only to certain locales.

APPROACH

At the time this study was undertaken, little data was available on the distribution and frequency of occurrence of pathogenic Naegleria in the environment. Many lakes and pools had been sampled and studied, particularly those responsible for outbreaks of PAM; however, usually only nonpathogenic Naegleria and/or Acanthamoeba were isolated. It was not until Griffin (8)

in 1972 suggested that temperature tolerance might be utilized as a selective force in isolating pathogenic Naegleria that epidemiological and environmental studies began to make considerable headway. Pathogenic Naegleria survived (and multiplied) at temperatures as high as 44-45°C; whereas nonpathogenic ones did not.

In 1975 DeJonckheere *et al.* (9) isolated pathogenic Naegleria from a thermally polluted canal in Belgium, the source of a fatal infection for a boy who had been swimming there. Subsequently reports began to filter in that pathogenic Naegleria could frequently be isolated from lakes in Florida (10).

Since it was well known that the Richmond, Virginia, area was highly endemic for PAM due to Naegleria, accounting for 17 cases, or more than any other geographic locale in the world, and since these cases had occurred over a time span of 10 years; it was believed that a study of lakes in this region of the country might aid in understanding the unpredictable appearance of PAM and the factor(s) or force(s) that might be responsible for the presence of pathogenic amebas. In addition, since the source for all previous cases of PAM in Richmond had been clearly traced to one of three fresh-water lakes located within a five mile radius, and since numerous other lakes in the locale had never been incriminated as a source of PAM, even though frequently used for swimming by the public, it was believed further that a careful study of the "non-PAM-related" lakes might yield valuable information if compared to data obtained from "PAM-related" lakes. Thus, nine lakes were selected for study for a variety of reasons (*vide infra*, see Section 4, MATERIALS AND METHODS, Sampling Sites). Included in the study were two lakes previously identified by patient histories as a source of PAM, namely, Moore's Lake and Lake Manchester. (The third lake, Lake Chester, associated with PAM in the locale could not be included since it had been closed and drained several years before by court order). In addition, two lakes (Lake Pocohontas and Pallisades Lake) were selected because pathogenic Naegleria had been previously recovered from their waters. Four lakes (Lake Salisbury, Swift Creek Reservoir, Falling Creek Reservoir, and Overhill Lake) were chosen because they had not been associated with the occurrence of PAM; and finally one lake (Lake Anna) was chosen because it was soon to become thermally enriched by the cooling effluent of a nuclear power plant (VEPCO North Anna Station).

Techniques of isolation and sampling for amebas were based on those successfully used by Dr. Flora Mae Wellings and her group at the Epidemiology Research Center, Tampa, Florida (10). The principle to be employed was temperature tolerance of pathogenic Naegleria; i.e., samples of water and bottom soil were to be incubated at 44°-45°C, with the belief that non-pathogenic amebas would be suppressed while pathogenic ones would survive. The methods of acquiring the samples would be filtering a large volume of lake water through a sterile sand column (with the belief that amebas would adhere to the sand particles), subsequently eluting amebas from the sand by a gentle detergent (beef broth), and finally harvesting the eluted amebas; and simultaneously collecting a small sample of soil from the surface of the bottom. Along with these protozoological studies, an analysis of the physicochemical (pH, dissolved oxygen, salinity, conductivity, and temperature)

parameters of each lake would be studied biweekly, as well as the concurrent climatological aspects of the locale. If possible, it was hoped that more extensive chemical analyses, as well as limnological studies, of the lakes could be obtained, perhaps through two nearby environmental groups at Virginia Polytechnic Institute and State University. In other words, every effort would be made to identify, characterize, and monitor the chemical and biologic properties of every lake under study with a particular effort to identify the differences and similarities which might serve as selective forces for the appearance or disappearance of pathogenic free-living amebas in nature.

SECTION 2

CONCLUSIONS

A 33-month study of the isolation of free-living amebas, especially pathogenic ones, from nine fresh-water lakes located in and around Richmond, Virginia, an area endemic for Primary Amebic Meningoencephalitis (PAM), and the relationship(s) of their isolation to a variety of physicochemical, bacteriological, and climatological factors revealed the following:

1. That ameboflagellates tentatively identified as Naegleria which could grow and survive at 44°C were present to varying degrees in every lake studied. Of a total of 1,311 samples of lake water and bottom soil tested, 16.2 percent were positive for such ameboflagellates.
2. That such ameboflagellates were isolated more frequently in the summer (the warmer months) than at any other time of year. Isolation of such ameboflagellates also varied widely from lake to lake (five percent for Lake Overhill to 33.8 percent for Lake Anna at its number 1 site). These findings were consistent with the seasonal (summer) appearance of PAM.
3. That free-living amebas which grew at 44°C but did not flagellate were isolated from 53.9 percent of the total 1,311 samples of water and bottom soil tested. The pathogenicity of these organisms was not tested and remained unknown.
4. That pathogenic Naegleria (i.e., N. fowleri) were present, but apparently in concentrations so small that their presence, especially in natural lakes, was detected infrequently. Of the 1,311 samples of water and soil examined, only 13 (one percent) contained pathogenic amebas, only five of which were from natural, nonthermally enriched lakes. These findings were consistent with the infrequent occurrence or rarity of the disease PAM in the local population.
5. That pathogenic Naegleria were isolated more readily from thermally enriched impoundments or lakes than from natural, nonthermally enriched lakes and that such impoundments or lakes through their artificially elevated water temperatures could serve as potential "reservoirs" for perpetuation and possible spread of such organisms to other lakes in temperate zones where such amebas are either non-existent or of little consequence. These findings might suggest

that these lakes will probably be a source of PAM in the future if man continues to use them for water sports or bathing.

6. That pathogenic Naegleria may not be present in deep waters but only in shallow waters (depending on the sensitivity of the test); as five pathogens were isolated from shallow water and eight from shallow bottom soil samples but none from deep water or deep bottom soil (as opposed to observations in Florida where deep sites were important and frequently positive).
7. That detection of pathogenic Naegleria increased significantly when water temperatures equaled or exceeded 30°C, especially when such water temperatures were sustained with little fluctuation over a period of 30 days or more. These findings appeared to explain, at least in part, the occurrence of PAM in this locale only in the warm months, particularly in the latter part of the summer.
8. That such pathogenic Naegleria were generally highly virulent, but occasionally apparently identical forms of lesser virulence could be isolated, the detection of which occurred only after subcultures of primary cultures of infected mouse brains were performed.
9. That often free-living amebas and/or ameboflagellates growing in Chang's liquid axenic media, a supposedly selective medium for pathogenic forms, were not pathogenic as determined by mouse-virulence tests. However, such growth, although initially good, could not be sustained, and such amebas eventually were lost in subculturing.
10. That pH, dissolved oxygen, salinity, and conductivity of lake waters appeared to differ little from lake to lake within the area under study, and furthermore within the ranges observed did not appear to be important determinants of whether or not pathogenic Naegleria could be isolated. However, pathogenic forms were notably absent from two swimming lakes (Overhill and Moore's) to which frequently were added large quantities of chlorine, potassium carbonate, and aluminum sulfate. Nevertheless, in the past one of these lakes (Moore's) has been associated with PAM (as judged from patient histories).
11. That isolation of pathogenic Naegleria from natural, nonthermally enriched lakes appeared to be less during the year 1979 following two severely harsh winters than during the years before (1977 and 1978), but this was not significant statistically. As expected, water temperatures of natural lakes varied directly with the ambient temperatures of the locale. These findings were operational in past epidemic years in which the disease PAM occurred in the resident population at risk, and supported the thesis that sustained elevations of water temperature were essential for detectable concentrations of pathogens to occur.
12. That the bacterial flora of the lakes studied may well have been

important in determining the population of pathogenic Naegleria present, and that such determinations appeared to depend on both qualitative as well as quantitative aspects. Several species, Serratia marcescens and Pseudomonas fluorescens, which were isolated from lake waters in large numbers, appeared to inhibit the growth of pathogenic Naegleria as determined by in vitro testing, and in some instances were toxic for them. Such findings may have explained the infrequent isolation of pathogenic forms.

13. That special extensive qualitative studies of the water and soil of the lakes under study indicated that highly enriched lakes (e.g., Lake Manchester) might not be associated necessarily with detectable levels of pathogenic Naegleria.
14. That these same special studies indicated that Lakes Anna I, II, and III, sites which were thermally enriched and which were associated with the greatest number of isolates of pathogenic Naegleria, contained high concentrations of iron and manganese, especially in their bottom soil. The significance of this was obscure, although it is known that pathogenic Naegleria are avid consumers of red blood cells (RBC), which contain iron in the form of hemoglobin, and their pathogenicity frequently can be revived by addition of RBC's to the culture medium.
15. That the strains of pathogenic Naegleria isolated were susceptible in vitro to the same agents and compounds and in the same concentrations as those which inhibit strains of N. fowleri isolated from humans dying from PAM. The polyenes were predictably amebicidal against environmental isolates of pathogenic Naegleria.
16. That culturing small samples of soil (about 500 gms) for pathogenic Naegleria by the methods outlined was easier, more economical, and more sensitive than culturing large volumes of water (72 L) and thus could be of practical use in future screening studies of fresh-water lakes. By the water sampling techniques employed, in order to isolate amebas, ameboflagellates, or pathogens required that at least one trophozoite per ten ml water be present. In undisturbed waters this concentration may be infrequent.
17. That prolonged incubation (up to 300 to 500 hrs.) of samples at 44°C appeared to enhance the isolation of pathogenic Naegleria, thus enhancing the sensitivity of the testing procedure and minimizing fears of transportation and storage losses.
18. That plankton nets were not acceptable for sampling lake waters for free-living amebas.

In summary these studies demonstrate that although the presence of pathogenic Naegleria in natural fresh-water lakes may depend on a variety of factors, water temperature, nutrients and bacterial flora appear to be the most important of the major determinants. High concentrations of iron and perhaps other metals or minerals may also be important, but this remains to

be proven. For natural fresh-water lakes, extremes of pH and excessive salinity (>0.5% NaCl) and/or conductivity adversely affect pathogenic Naegleria, but depressed levels of dissolved oxygen may not be as critical as once believed despite the aerobic nature of these organisms.

Since isolation of pathogenic Naegleria from fresh-water lakes, along with determination of factors affecting the appearance of pathogens, were the objectives of this study, the area under study proved to be excellent. It had been associated frequently with human PAM, and pathogenic Naegleria were present only periodically and not in all lakes; so that when pathogens were isolated, their presence could be studied and correlated with variations in physicochemical or biological changes or pressures.

SECTION 3

RECOMMENDATIONS

Based on the data obtained in this study and that reported by others the following recommendations are made:

1. That all fresh-water lakes whose waters may reach or exceed 30°C and which are utilized for public swimming should be assessed for their content of pathogenic Naegleria and for their ability to support such organisms. This may be accomplished by utilizing the methods of analyzing bottom soil described in this report, which are simple, economical, and reasonably reliable. The water itself need not be cultured.
2. That if pathogenic Naegleria are isolated from a fresh-water body of water used for swimming, the lake should not be opened to the public when water temperatures exceed 30°C, and that authorities should be made cognizant of the fact that with each day such water temperatures exceed 30°C, the concentrations of pathogenic Naegleria may be increasing, and that if man is exposed to such waters in any intimate way (e.g., swimming), the risks of developing PAM may be heightened.
3. That if pathogenic Naegleria are isolated from a lake, further studies should be undertaken to determine the ease with which this can be accomplished (e.g., sequential sampling and, if possible, quantitative studies to determine the concentration of such pathogens, perhaps employing dilutional or MPN methods). Thus, the risks in terms of exposure to pathogenic Naegleria should be assessed.
4. That thermal enrichment of lakes containing pathogenic Naegleria should be discouraged, limited, or controlled and that public swimming in such lakes or impoundments should not be permitted.
5. That the Lake Anna system receive continual surveillance for pathogenic Naegleria, quantitatively as well as qualitatively, and that the residents in that locale be made aware of the threat that might exist if bathing or swimming is pursued while thermal enrichment exists.
6. That the addition of chemicals (e.g., NaCl, aluminum sulfate, and halides) to fresh-water lakes be studied as to their effect on

pathogenic Naegleria, as well as on the competitive protozoan flora and other microbes. It is possible that such chemicals may be detrimental, as well as beneficial.

7. That potable water used for consumption and bathing and obtained from reservoirs be particularly scrutinized for pathogenic Naegleria, and that filters, piping systems, and the end product itself be studied for the presence of such forms.
8. That epidemiologic studies on human populations using fresh-water lakes for recreation be undertaken to determine the attack rates of pathogenic Naegleria and to determine if subclinical or nonfatal infections, meningitis or otherwise (e.g., respiratory) are occurring. This may be done by studying human sera and measuring antibodies against the resident pathogenic Naegleria isolated from lakes in the locale.
9. That bacteria (and possibly fungi and protozoa) be more carefully studied for their effect (interference or enhancement) on the appearance of pathogenic, as well as on nonpathogenic amebas (especially Naegleria).
10. That the interrelationships between pathogenic Naegleria and other protozoan species (competitors and predators) be more carefully explored than has been done in the past.
11. That the effect of iron and other metals or minerals on the appearance of pathogenic Naegleria be studied.
12. That microcosm studies be performed in model systems in which variables may be separated and individually tested in the laboratory to quantitate their effect and to study their interrelationships.
13. That pursuit of possible factors which might induce nonpathogenic Naegleria to become pathogenic forms continue to be explored.
14. That other selective methods for pathogenic Naegleria (e.g., media containing inhibitory agents for non-pathogenic amebas) be sought, as the use of elevated temperatures (44°C) appears to be too non-specific and growth in Chang's liquid media not always dependable.
15. That free-living amebas growing at 44°C which are nonflagellating be further studied for pathogenicity, as there is an increasing number of fatal cases of PAM being reported from species other than N. fowleri.
16. That the natural spread or dissemination of pathogenic Naegleria or the methods whereby lakes may be seeded with such forms should be studied.

SECTION 4

MATERIALS AND METHODS

SAMPLING SITES

A total of nine fresh-water lakes, all within a 27-mile radius and located in the Richmond, Virginia, area were sampled and studied during a three-year time period extending from October 1, 1976, through September 30, 1979. The lakes studied were: Anna, Salisbury, Pocahontas, Manchester, Falling Creek, Moore's, Pallisades, Overhill, and Swift Creek (see TABLE 1 and Figure 1). Lake Anna was sampled at three different points (referred to as Lakes Anna I, II, and III). In the second and third year of the study, Lakes Salisbury, Falling Creek, Swift Creek, and Pocahontas were sampled both in deep and shallow locations. An adjacent feeder lake for Overhill also served on occasion as a sampling source.

Most lakes were sampled continually, usually biweekly, throughout the study period; while others were sampled periodically or over a shorter than three-year period for reasons beyond the control of the investigators (e.g., owners refused to cooperate; lakes were drained; etc.). Selection of lakes was based on a variety of factors: (1) location in an endemic area for PAM; (2) a past epidemiologic history of being the source of one or more fatal cases of PAM; (3) a past history of never having been incriminated as a source of PAM, despite close proximity to lakes which had; (4) past history of having a pathogenic strain of *Naegleria* isolated either from the water or from bathers; (5) frequent use of the lake(s) by the public as a bathing and/or recreational area(s) for water sports; (6) use of the lake as a potable water supply for the local community; (7) vast differences in chronological ages of the lakes and apparently in eutrophy; (8) subjugation of lake to thermal pollution; (9) geographic proximity (i.e., for logistical reasons); and (10) cooperation of owner(s) or authorities, thus permitting sampling. The following is a brief description of each lake studied.

Lake Anna

This 17-mile long, 9,600 acre, fresh-water lake with 200 miles of shoreline is located on coordinates 77°47'30" and 38°3'30" about 40 miles north by northwest of Richmond and 24 miles southwest of Fredericksburg, Virginia, between the counties of Orange (to its northwest), of Louisa (to its south) and of Spotsylvania, to its north and northwest (see Figure 2). Its creation dated back to January 10, 1972, when a mile-long dam across the North Anna River (flowing west to east) was plugged as part of the development of the Virginia Electric and Power Company's (VEPCO) "North Anna Power

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TABLE 1. NAME OF VIRGINIA LAKES
(AND LOCATION OF SAMPLING)
WITH DESIGNATED SITE NUMBER
(SAMPLING PERIOD DEC. 3, 1976-AUG. 14, 1979)

Name of lake (with location of sampling)*	Designated site number
Overhill (shallow)	1
Overhill (feeder lake)	14
Anna I (shallow)	2
Anna II (shallow)	3
Anna III (shallow)	4
Pocahontas (shallow)	5
Pocahontas (deep)	18
Salisbury (shallow)	6
Salisbury (deep)	13
Manchester (shallow)	7
Falling Creek Reservoir (shallow)	8
Falling Creek Reservoir (deep)	17
Moore's (shallow)	9
Moore's (deep)	19
Pallisades (shallow)	10
Swift Creek Reservoir (shallow)	11
Swift Creek Reservoir (deep)	12

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* Shallow defined as 3 to 6 inches. Deep: see details of Materials and Methods.

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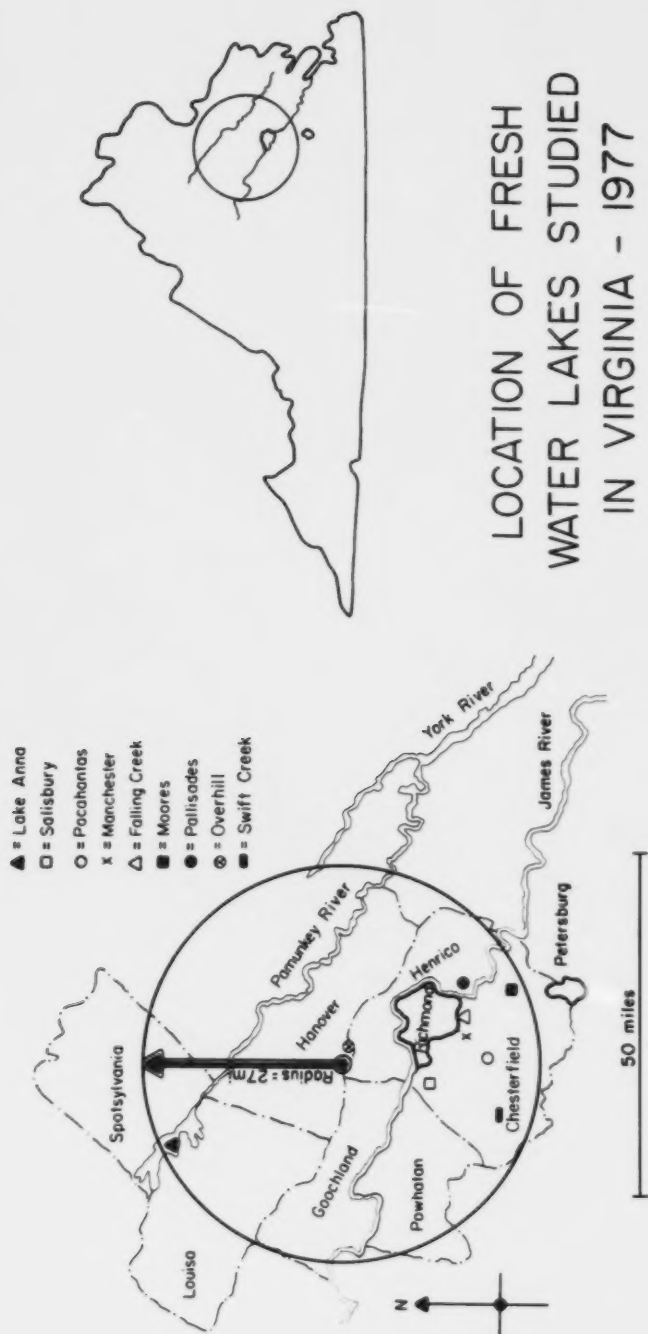


Figure 1. Map of Richmond, Virginia, area and location of nine lakes sampled.

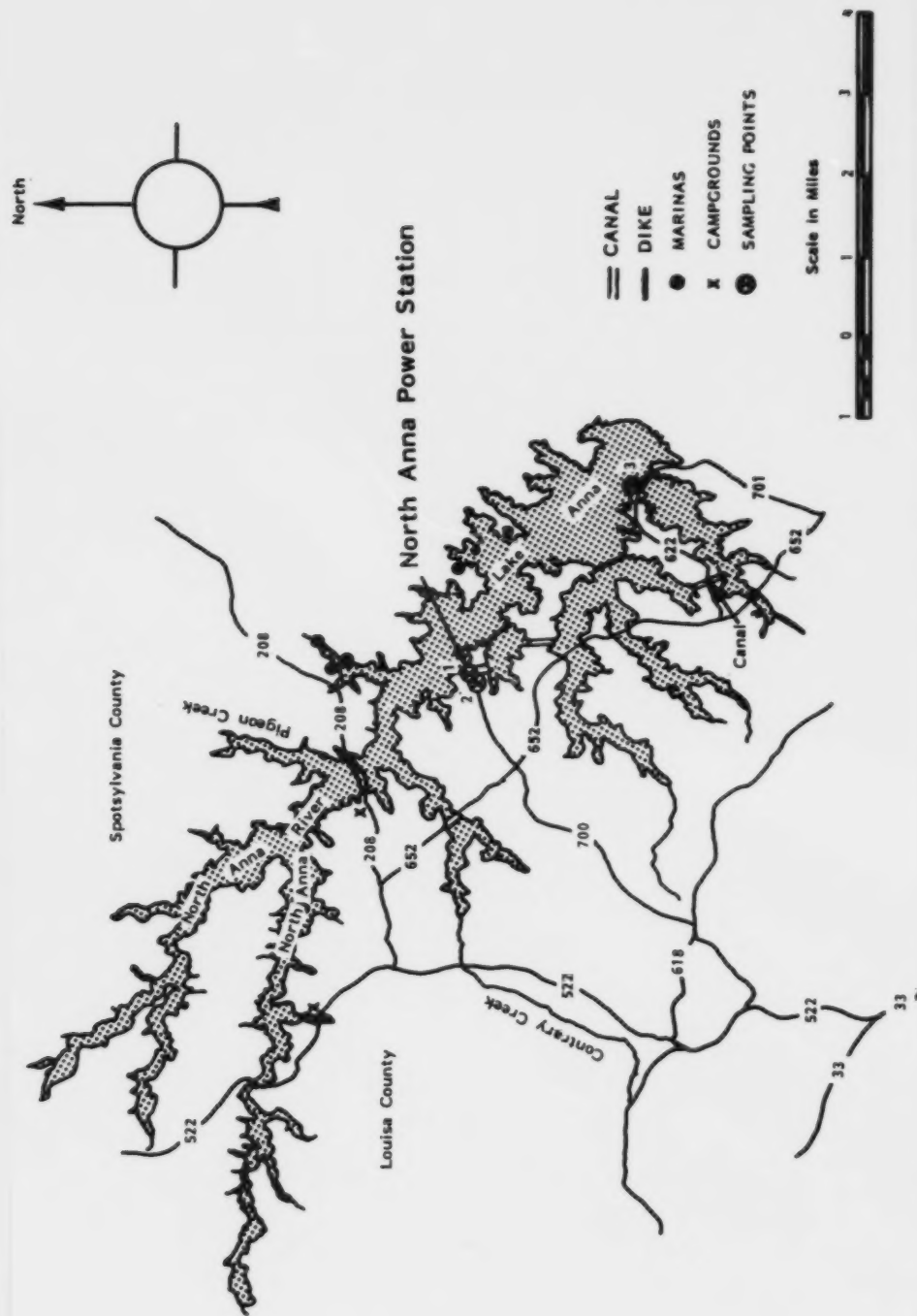


Figure 2. Map of Lake Anna, located north of Richmond, Virginia. Note locations of North Anna Power Station (1) at Lake Anna I beside the thermal plume; the sampling site on Lake Anna II (2) or on the banks of the first coolant canal; and the sampling site on Lake Anna III (3) just beside the last dike separating it from the final coolant lagoon. Note also the location of Contrary Creek which empties into the main reservoir but not into the coolant lagoons.

Anna II (see Figures 3 and 6).



Figure 3. Aerial view of a part of the Lake Anna Reservoir (above) showing VEPCO North Anna Station and the first coolant canal. The arrows point to sampling sites 2 and 3 for Lakes Anna I and II, respectively, along the canal.



Figure 4. North Anna Nuclear Power Station.

This sampling site (numbered 3) was located approximately 0.65 miles away from Anna I, or the first sampling site (vide supra), and was situated along the banks of the first coolant canal. The bottom soil was also loamy, and the immediate area was surrounded by light underbrush and weeds. The sampling depth was approximately four to six inches.

Anna III (see Figures 7 and 8).

This sampling site (numbered 4) was located approximately 4.5 miles away from Anna I (vide supra) and was along the shores of the main reservoir, which itself was contiguous with the third coolant lake and its outlet but separated by a dike. The bottom soil was loamy and of the same consistency as Anna I and II. The sampling depth was approximately four to six inches.

The main reservoir was a relatively shallow (mean depth 25 feet), relatively warm, monomictic (October was turnover time), main stream impoundment, which remained generally free of ice (exception: winter of 1978-79). At the onset of this study in 1976, the reservoir was characterized as mesomorphic with a paucity of blue-green algae. The lake and its coolant lagoons were formed by the North Anna River, which has its origin on the eastern slopes of the Blue Ridge Mountains in Orange County. The river flowed eastward over the Piedmont Plateau into the York River Basin. Its water was described as medium hard, carrying a pH of approximately 7.0. The dam, with a minimum flow of 40 cu. ft. per sec., maintained a fairly constant water level for the reservoir.

Flowing into one of the main arms of the Lake Anna reservoir from the south was Contrary Creek, of interest because it contained a number of acid mines, which resulted in a pH of approximately 3.0 at its mouth and which was not fully diluted out until about one kilometer (personal communication through Dr. William Odom, University of Virginia Department of Environmental Sciences, August, 1979). The water from Contrary Creek also contributed sizeable amounts of iron, sulfates, and copper, as well as possibly zinc, manganese, lead, and cadmium. The soluble forms of these heavy metals appeared to be diluted out by the time the main body of the reservoir was reached. Bacterial counts of sediment and water appeared to be reduced in Contrary Creek and at its mouth; and of particular interest was that each spring large fish kills had been noted in this region, apparently following periods of runoff which were highly acid. The temperatures at the mouth of Contrary Creek were often as high as 29-30°C during the months of July, thought to be due to warming from the sun over large shallow areas.

Salisbury Lake (numbered sampling sites 6 and 13)

This lake was located in Chesterfield County about five miles west of Richmond with coordinates 77°39' and 37°31'30". It was in a wooded, heavily populated, suburban area and was about two miles long and one-half mile wide. It was created in 1975 by damming up a number of springs in the locale. The lake was frequently the source of runoff of organic phosphates and fertilizers from a nearby golf course.



Figure 5. Site #2 (Lake Anna I) beside thermal plume at discharge site of North Anna Station.



Figure 6. Site #3 (Lake Anna II) along end of first coolant canal. Notice ground cover.



Figure 7. Aerial view of Lake Anna III or site #4 (arrow) along dike separating last coolant lagoon (right) from main reservoir (left).



Figure 8. Lake Anna III (site #4). Note dike (right).

The lake was used only sparingly for water recreation; however, children frequently swam there. The sampling site was located on a sandy shore containing weeds. The sampling depth was three to six inches at a point where the bottom was rocky clay and plant life was visibly present. Deep samples located about 100 feet out from the shallow sample site were taken at about six feet; here the bottom was muddy and clay-like.

No cases of PAM had ever been traced to this lake. It was chosen for study because of its location in a locale endemic for PAM, i.e., Chesterfield County; for its "newness"; and for logistical reasons because of its accessibility and the cooperation of its owners.

Falling Creek Reservoir (numbered sampling sites 8 and 17)

This lake, located in Chesterfield County just south of Richmond with coordinates 77°28' and 37°21'30", was created in 1952 as an impoundment. Its source of water, Falling Creek, had its origin in Chesterfield County from various springs and from Pocoshock and Pocosham Creeks. The reservoir, located in a wooded area containing many private homes along its shoreline, contained 302 million gallons and had a surface area of 88.67 acres. It was about two miles long and one-half mile wide and drained an area of 38 square miles. Swimming and boating were moderately practiced within it. The lake was considered to be much more eutrophic than nearby Swift Creek Reservoir (vide infra). A shallow sampling site was situated on a grassy shore with a water depth of four to six inches. The water was clear, and the bottom was rocky with much visible plant life. Samples were taken also from a deep site, about 18 feet, and located 20 feet off shore; here the bottom was muddy.

Falling Creek Reservoir had never been directly incriminated as a source for PAM; however, its water source probably was identical to that of two nearby small lakes that were directly and clearly associated with PAM in the past. In addition, several histories of patients dying from PAM suggested that Falling Creek Reservoir may have played a role as the source of their disease. Finally, the lake served as the potable water supply for a part of Chesterfield County.

Manchester Lake (numbered sampling site 7, see Figure 9)

This lake was located about a mile west of Falling Creek Reservoir with the coordinates 77°31' and 37°29'. It was a small, less than an acre in size lake, which was no longer used for recreation. An apartment complex was adjacent to the lake. It was situated in a business district next to U. S. Highway 360. The sampling site was along a grassy shore in three to six inches of water. The bottom consisted of sand and rocks, and plant life was easily visible in clear water. It did not contain fish. The lake was chosen for study because it was clearly associated with a fatal case of PAM (in the summer of 1967 (11)).

Swift Creek Reservoir (numbered sampling sites 11 and 12)

This lake was located in Chesterfield County eight miles southwest of

Richmond. It represented a 1,700 acre impoundment on Swift Creek that contained approximately 5.2 billion gallons of water and served as a major water supply to Chesterfield County. The reservoir had a rural drainage basin of 65 square miles (41,120 acres). Approximately 85 percent of the reservoir's drainage basin was in Chesterfield County. The remaining 15 percent was in eastern Powhatan County. The reservoir was constructed in 1966. The spillway was a concrete, broad-crested weir, approximately 320 feet wide and set at an elevation of 177.2 feet above mean sea level (msl). In 1967, the Swift Creek Water Treatment Plant (WTP) was completed. Although the reservoir theoretically provided 12 million gallons of water per day (mgd) without supplementary supply, the treatment plant had a daily capacity of 10 mgd. Swift Creek reservoir was also a site of limited, passive recreation, principally fishing and boating. The reservoir had been designated a "critical environmental area" by the State of Virginia. The State indicated that the reservoir was a valuable natural area with significant recreational potential in the midst of an urbanizing region.

Since its construction in 1966, Swift Creek Reservoir had exhibited some symptoms of eutrophication, including periodic algal blooms, anoxia in the deeper portions of the reservoir, and the appearance of nuisance growths of pondweed (*Potamogeton* sp.). The average yearly total coliform on the raw water had been described as approximately 30 MPN.

The shallow sampling site was situated along a grassy shore in three to six inches of water. The bottom was sandy and contained much visible plant life; the water was a clear brown. Deep samples were obtained also at a depth of eight feet from a site about 20 feet out from the shallow site. The bottom at this location was muddy.

Swift Creek Reservoir was chosen because it was located in Chesterfield County, an endemic locale for PAM; it was the source of water for several lakes previously associated with PAM; it supplied the potable water for Chesterfield County; and it served as a growing recreational area for water sports in the area.

Pocahontas Lake (numbered sampling site 5 and 18, see Figure 10)

This lake was located in Pocahontas State Park in Chesterfield County about ten miles south of Richmond with coordinates 77°36'30" and 37°23'30". The lake was in a sparsely populated, wooded area and was about one mile long and one-fourth mile wide. It was used by the public for fishing, swimming, and canoeing.

In the first year of the study only shallow samples at depths of three to six inches were obtained; however, in the latter half of the study deep samples (seven ft.) about 20 ft. offshore were secured. The shallow sampling site was along a grassy shore, and the water was consistently a mahogany brown in color. It contained much visible plant life. Both bottoms were sandy and/or loamy.

Although no cases of PAM were ever traced directly to Lake Pocahontas, it was chosen for study because it was located in Chesterfield County, an



Figure 9. Manchester Lake (site #7). Note surrounding apartments.



Figure 10. Lake Pocahontas (sites #5 and 18). Note dense forest surrounding lake (site of State park).

endemic locale for PAM, and within five miles of two lakes which had earlier been incriminated as a source of PAM. In addition, in the summer of 1968, an epidemiologic study had been conducted by the Center for Disease Control (Atlanta) in which nasal cultures from 155 children who had been swimming in the lake were performed in search of pathogenic Naegleria (one such isolate was obtained) (12).

Lake Overhill (numbered sampling sites 1 and 14)

This lake was man-made, about 100 feet by 50 feet and contained cement sides and a sand bottom. It was frequented by the public and was used for swimming and diving. The lake was located in a sparsely populated, wooded area in Hanover County about ten miles north of Richmond with coordinates 77°33' and 37°41'. Lake Overhill was filled from an adjacent "feeder lake" which was itself spring-fed. The water was filtered by a makeshift system, and during the swimming season (summer), a number of chemical disinfectants (chlorine), and aluminum sulfate and potash were added. Samples were taken at depths of four to six inches along the swimming lake at a point where the water was generally clear and green. There was no grossly observable plant life at this site. Occasionally the "feeder lake" was sampled at depths of four to six inches. This water was red colored and contained much grossly visible plant life. Its shoreline was grassy and treelined; and the bottom was sandy. Overhill "feeder lake" was about 200 feet by 100 feet in width. Fishing was the only recreation carried on within it.

Moore's Lake (numbered sampling sites 9 and 19, see Figures 11 and 12)

This lake was located in Chesterfield County about six miles south of Richmond with the coordinates 77°24' and 37°21'30". It was used for public swimming and had cement sides and a sandy bottom. It contained diving platforms, sliding boards, and many wooden piers. In the middle of this 100 ft. x 100 ft. lake were fountains which aerated the water. Trees surrounded the lake, and a trailer complex was next to it. Site #9 was located at a water depth of two feet. There was no visible plant life, the bottom was sandy, and the water was generally a murky green. Site #19 was the deep site, where the water was eight feet deep. As with Lake Overhill (vide supra), chemicals were added.

This lake was chosen because it had previously been incriminated as a source of PAM, because it was nearby to a lake which had since been closed but had been associated with several cases of PAM, and because it was frequented by the public as an area for bathing.

Pallisades Lake (numbered sampling site 10)

This lake was located in Chesterfield County about three miles south of Richmond with coordinates 77°23'45" and 37°26'. It was a swimming lake containing diving platforms and slides. There were no cement sides, and the shores and bottom were clay. The size of the lake was about 150 ft. x 150 ft. and it was located in a rural area with a scarcity of surrounding trees. Sampling was at a water depth of two to four inches; there was no plant life visible, and the water was generally a clear brown.



Figure 11. Aerial view of Moore's Lake. Note surrounding foliage.



Figure 12. Moore's Lake (sites #9 and 19)

This lake was chosen for study because of its proximity to other lakes which had been incriminated as being a source of PAM, yet it had not been mentioned in epidemiologic histories as a possible source; its use as a public swimming lake; and the fact that an earlier study conducted by this laboratory in 1974 had obtained a pathogenic isolate on one occasion (confirmed by S. L. Chang, unpublished data).

STOCK CULTURES

Stock cultures of amebas used in this study included several originally isolated from fatal human cases of PAM (C. J., D. D., G. J., L. L., R. N., T. Y., W. M., '66, '69, #359, #838), and an environmental pathogenic isolate (L. Monroe). Each culture of a pathogen was maintained axenically in Chang's liquid calf serum yeast-extract-casein-medium (CSYECM) at room temperature (23°C) and subcultured for passage into Chang's CSYECM every two weeks. The stock cultures of flagellates were maintained on "flagellate plates" (see APPENDIX A-7) at 4°C and transferred to new plates every four months. Survival at this temperature was excellent.

ISOLATION OF AMEBAS

Techniques of Sampling (See APPENDIX A-4 and Figures 13 through 15)

Sampling was conducted generally on a biweekly schedule for most lakes. Water samples were obtained by pumping water through a 61 cm column (inner diameter 3.33 cm) filled with Ottawa #20-30 sand. The pump used was a 12-volt Teil marine-utility pump (model IP S80B), which was run by a 12-volt Sears Die-Hard marine battery. Depending upon the flow rate at each site, the pump was left on long enough for 72 liters to run through the column filter. At all sites, water was pumped from a point four to six inches off the bottom, not from the surface, and over an undisturbed area of bottom soil.

Bottom soil samples from shallow sites (three to six inches) were taken with a long flat spatula. About 500 gms of the upper one-half inch portion of the bottom soil were carefully removed from an area of six square centimeters and placed in a plastic zip-lock bag. Bottom soil samples from deep sites were taken by dragging metal cylinder 23 cm long with a diameter of ten cm along the bottom. The collecting cylinder opened at one end and had holes in the opposite; so that particulates of soil entered through the open end and water drained out through the opposite. The soil was placed in zip-loc bags and immediately placed in a cooler to be transported to the laboratory for processing.

Processing of Samples (See APPENDICES A-5 and A-6 and FLOW SHEET on page 28)

All samples were taken in the morning and were brought to the laboratory that afternoon for processing. Approximately 500 gms of bottom soil was brought in plastic ziploc bags and then incubated overnight for 18 hours at 44°C. A 61 cm column containing Ottawa #20-30 sand, through which 72 liters of lake water had been filtered, was incubated on its side overnight for 18 hours at 44°C. The next day both the column sample and bottom

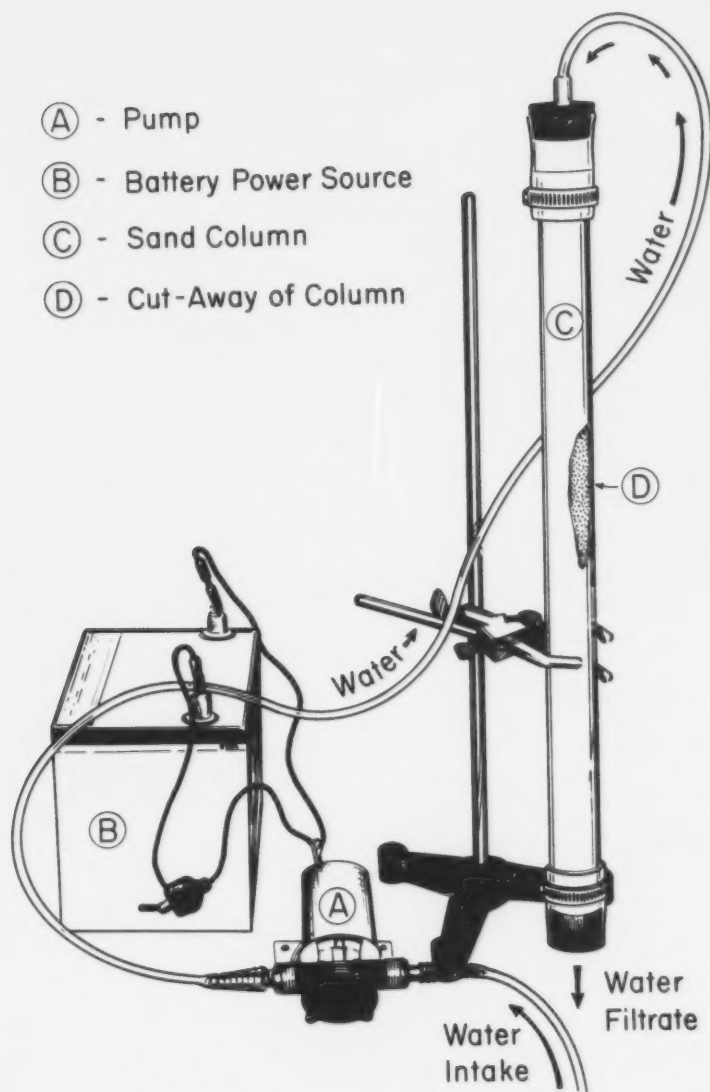


Figure 13. Apparatus used for filtering water to detect free-living amebas. A. 12 volt Teil marine utility pump. B. 12 volt Sears Die-Hard marine battery. C. Teflon cylindrical column 61 cm high and 3.33 cm wide. D. 45 cm high column of #20-30 Ottawa sand.

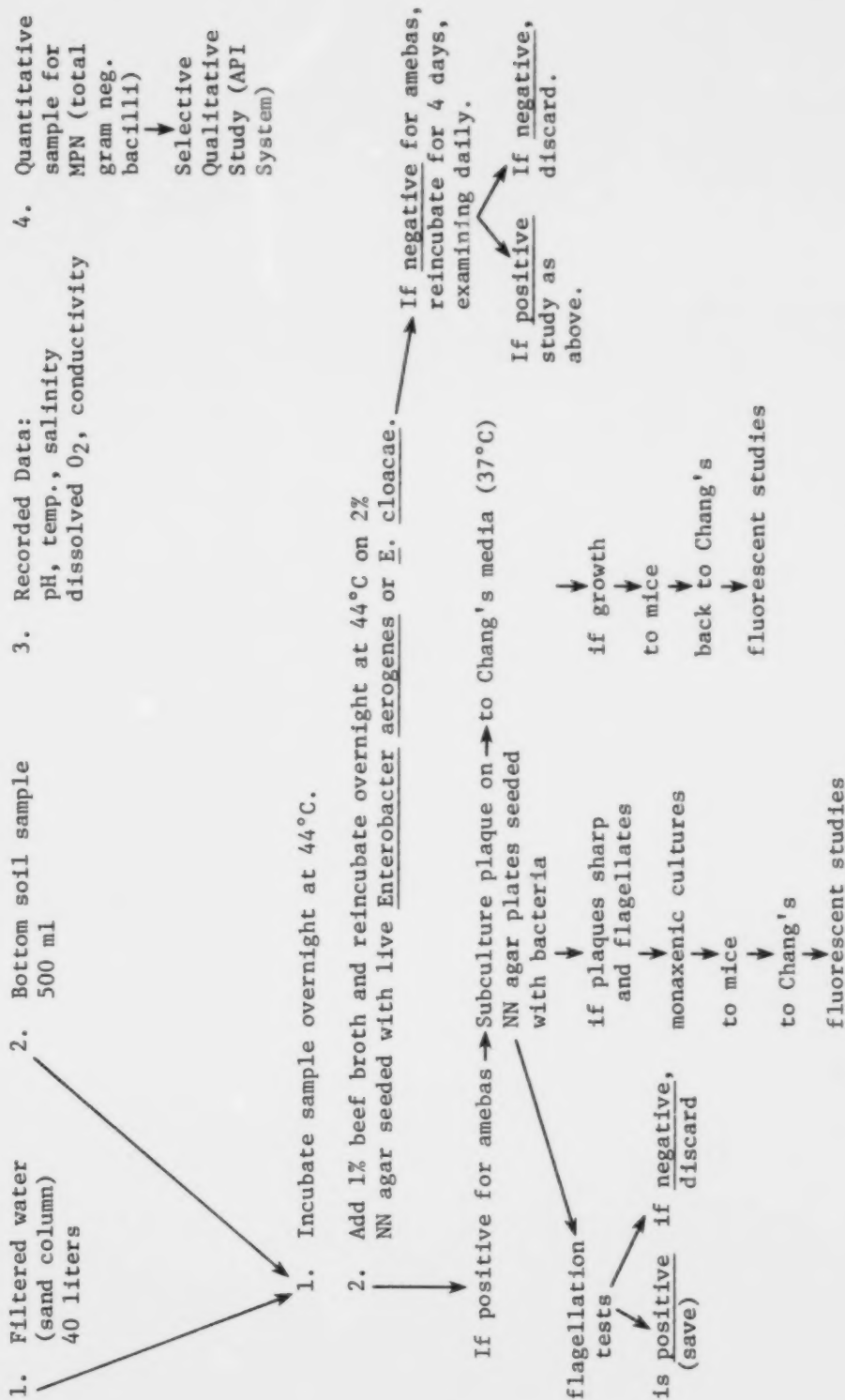


Figure 14. Use of water sampling apparatus in the field.



Figure 15. Obtaining bottom soil in the field.

FLOW SHEET FOR STUDY OF FRESH WATER LAKES
FOR PATHOGENIC FREE-LIVING AMEB/1.3



soil samples were emptied separately into sterile one liter plastic containers.

To the water column sample was added 250 ml of one percent beef extract broth (Difco #0126-01). The container was shaken vigorously for three minutes, and the sand was allowed to settle. The fluid was decanted into a sterile 250 ml centrifuge bottle and concentrated by centrifuging at 959 x g for 30 minutes at 4°C on a Beckman J-21C Fixed Rotor. The supernatant was gently poured off leaving a small suspension, which was poured into a #2070 conical centrifuge tube and centrifuged at 614 x g for 30 minutes at 4°C. The remaining supernatant was poured off, and the pellet vortexed vigorously for five seconds. This pellet was used to inoculate nonnutrient agar plates seeded with live Enterobacter cloacae (referred to as "monaxenic plates").

To the bottom soil sample was added approximately 100 ml of one percent beef extract, which was shaken vigorously for three minutes, then decanted into a 250 ml centrifuge bottle. Approximately 100 ml beef extract broth was added to the one liter container which was shaken vigorously again for three minutes and decanted into a second 250 ml centrifuge bottle. These containers were centrifuged at 959 x g for 30 minutes at 4°C, and the fluid was decanted, and the sediment was used to inoculate monaxenic plates.

Culture Techniques (See APPENDICES A-1, A-2, and A-3)

Monaxenic. Petri plates with a two percent solution of nonnutrient agar (Difco #0560-01) were made up in the laboratory. In order to be easily read through the inverted microscope, the depth of the agar was approximately 5-7 mm. Live E. cloacae, an environmental isolate from Lake Anna I, was shown to be an excellent food source for amebas in a study using a pathogenic environmental isolate #931, in which plaque diameters were measured and observed macroscopically and microscopically for healthy, motile trophozoites. The E. cloacae was subcultured daily to nutrient agar (Difco #0001-01) and incubated at 37°C for 18-24 hours. Each day it was checked for purity, and bimonthly this bacterium was checked for biotype according to the API (ANALYTAB PRODUCTS) system for identification of Enterobacteriaceae. The seeding of plates involved taking a 24-hour culture of E. cloacae, flooding it with 5 ml of sterile distilled water, and pipetting it into a sterile test tube. A suspension was created from which 0.2 to 0.3 ml were pipetted onto the center of a nonnutrient two percent agar plate. Using a glass rod dipped in alcohol and flamed, the suspension was spread evenly over the agar and allowed to dry. The vortexed pellet from the column sample was dropped or poured onto two such plates, and bottom soil samples were inoculated onto the center of six such plates using a sterile cotton swab. All plates were inverted and incubated at 44°C.

Axenic. Axenic cultures of Naegleria fowleri were maintained in Chang's liquid axenic medium (CSYECM) (see APPENDIX A-1). The medium was dispersed aseptically in 5 ml amounts into 16 x 125 screw-capped tubes, checked for sterility by incubating at 37°C for 48 hours, and refrigerated at 4°C until used. A control tube with N. fowleri was set up and incubated at 31°C in which luxuriant growth should have been present by 48 to 72 hours. Nelson's media (see APPENDIX A-3) was used intermittently in main-

taining axenic cultures. This media was stored in 100 ml screw-capped containers. Quality control and sterility were performed the same as with Chang's medium.

Stocking and Storage

Stocks of axenic N. fowleri and ameboflagellates were maintained. N. fowleri was grown in Chang's axenic medium (see APPENDIX A-1) or Nelson's media (see APPENDIX A-3). Every two weeks stock cultures were vortexed vigorously and 0.5 ml of stocked Naegleria were transferred to 5 ml of fresh media, either Nelson's or Chang's. These cultures were incubated at 37°C for 48 hours, checked for luxuriant growth, and then stored at room temperature until the next transfer.

Ameboflagellates were transferred directly from the flagellate plate on which they were found to another flagellate plate (two percent nonnutrient agar made with Page's Saline (see APPENDIX A-2). This transfer involved cutting out a plug of agar and placing it face down on another agar plate. This plate was then incubated for 18-24 hours at 44°C, checked for growth, and inverted and stored at 4°C. Every four months stock cultures were checked for growth and transferred to new plates.

IDENTIFICATION OF AMEBAS

All environmental samples on monaxenic plates seeded with live Enterobacter cloacae were incubated for 48 hours at 44°C. After 48 hours, plates were observed macroscopically for sharp plaque lines and a clean area between the sample and plaque line, indicating a reduced number of bacteria (see Figure 16). Plates were observed under an inverted microscope at 250x magnification. Trophozoites were identified as being approximately 10-12µ in diameter, and as having eruptive broad pseudopods or long and slender ones with spiny projections. Cysts were smaller than trophozoites, rounded in appearance, contained double walls, and were without movement. Cysts and trophozoites were found clustered near the sample and near the outer plaque lines; but they were sparse between the sample and outer plaque line. One centimeter square agar plugs were removed between the sample (to avoid environmental bacteria) and the plaque line (to avoid the seeded E. cloacae outside the plaque line). Plugs were subcultured onto two percent nonnutrient agar seeded with live E. cloacae. Two separate subcultures were made from the water column sample and six separate ones from the bottom soil sample. Each was inverted and incubated at 44°C for 24-48 hours and subsequently examined for plaque lines and amebic growth.

All subcultures were examined at 24 hours for plaque lines and subcultured again to test for flagellation and growth in Chang's liquid medium (see FLOW SHEET on page 28). Flagellation plates (50 x 9 mm containing nonnutrient agar and Page's saline - see APPENDIX A-7) were seeded with live E. cloacae. A centimeter square plug was placed in the center of two flagellate plates, inverted, and incubated for 18-24 hours. Plates were examined for plaque lines, flooded with sterile distilled water, and incubated at 44°C. The plates were then examined on an inverted microscope at one and one-half to two hours and throughout the day for the presence of

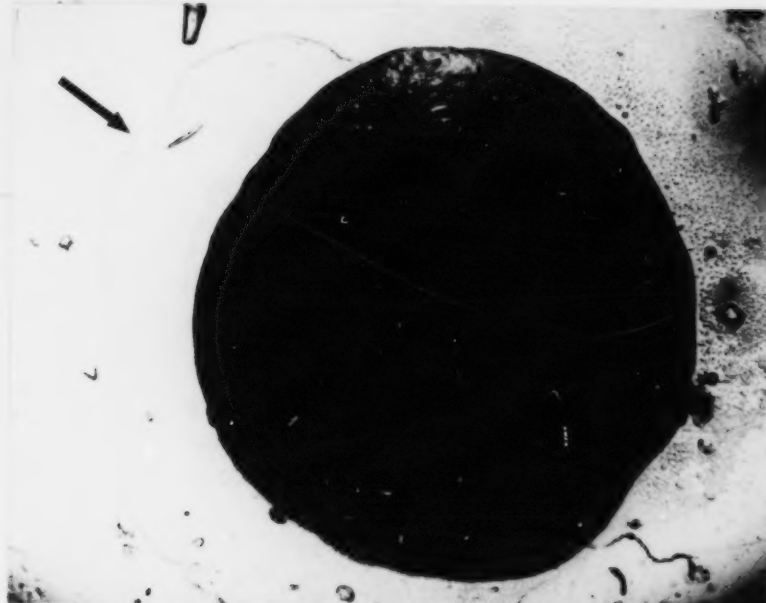


Figure 16. Example of plaque formed by amebas 24 hrs. after incubation of a bottom soil sample at 44°C. Arrow points to edge of plaque line.

flagellates. Flagellates were identified as amebas that slowly rounded up (ameboflagellates) and became spherical as the anterior flagellum outgrowth began. The cells began to rotate at an accelerating rate and soon swam free as they gradually elongated into a characteristic elliptical flagellate shape. A monaxenic culture plate of each ameboflagellate was stocked and maintained at 4°C.

During the first year of the study, only those isolates which would flagellate were inoculated in Chang's CSYECM; however, in the second and third year of the study for reasons of simplicity, at the same time the sample was subcultured to test for flagellation, it was subcultured into Chang's liquid axenic medium. It was incubated at 37°C, and checked daily for growth up to 72 hours. Growth was described as a monolayer of healthy, motile trophozoites having characteristic broad eruptive pseudopodial movement.

PATHOGENICITY STUDIES (See APPENDIX A-8 and FLOW SHEET on page 28)

If growth of amebas occurred in Chang's CSYECM, mice were inoculated to test for pathogenicity. Concentrates of the amebas and a control of N. fowleri (W. M. strain), both 48-72 hours old, were centrifuged at 614 x g for 15 minutes at 4°C. The supernate was removed with a sterile pipet, and 1 ml of Chang's CSYECM was added and vortexed for five seconds. The concentration or number of amebas per ml was calculated using a hemocytometer

counting chamber and appropriate adjustments were made to yield an inoculum of approximately 10^4 trophozoites per 0.01 ml.

To test for pathogenicity, three week old DUB/ICR (Dublin, Va./International Cancer Research) male mice were lightly anesthetized with ether, and a 0.01 ml suspension of amebas was instilled intranasally into each of five or ten mice. Mice were checked daily for 21 days; moribund mice were sacrificed by cervical dislocation. The brain and olfactory bulbs of the mice were observed for hemorrhage, removed, and cultured on a small nonnutrient agar plate and in Chang's CSYECM. The nonnutrient agar plate was observed directly for the presence of amebas. The percent mortality due to amebas was recorded. An axenic culture of all pathogenic isolates was stocked and maintained in Chang's CSYECM at room temperature.

PHYSICOCHEMICAL STUDIES

Temperature. Temperatures were obtained with a YSI model 33 S-C-T meter and recorded in °C. The probe was placed on the bottom of the lake at the sampling site during the time of sampling. The meter gave a readout of -2°C to 50°C. Periodically, the meter was compared with a mercury thermometer; it remained comparable at $\pm 0.3^\circ\text{C}$.

pH. A sample of 250 ml of water was collected from the pump at each site and returned to the laboratory in an ice chest. Once in the laboratory, the water was allowed to settle at room temperature, and within an hour the pH was measured using a Corning Portable pH meter (model 610 A).

Dissolved Oxygen. The Winkler Titration Azide modification as outlined in Manual of Methods of Chemical Analysis of Water and Wastes, EPA-625/6-74-003, was used to determine the amount of dissolved oxygen (D.O.) in the water at each sample site. Water was collected in a 300 ml glass bottle directly from the pump, care being taken not to let air bubbles into the bottle. To this sample was added 2 ml of manganese sulfate solution (364 gm Mn SO₄H₂O in 1.0 L H₂O). Next, 2 ml of alkali-iodide-azide reagent was added well below the surface and the bottles shaken 15 times to give the hydroxide ions an opportunity to combine with the iodide ions. When the precipitate settled, the bottle was shaken again and allowed to sit for two minutes. Two ml of concentrated H₂SO₄ was gently added, and the bottle shaken until dissolution was complete. After dissolution, 97 ml was poured off; so that 203 ml were left, which corresponded to 200 ml of the original sample after correction for the loss of sample by displacement with the reagents. Starch (2 ml) was then added, forming a complex with the iodine to turn the solution blue. The iodine was titrated with 0.0250 N sodium thiosulfate until the blue color disappeared. The amount of titrant added corresponded to the amount of D.O. present in the sample and was recorded in parts per million (ppm).

Salinity and Conductivity. These parameters were also measured using the YSI meter (model 33 S-C-T), the same one used for temperature measurements. Salinity was defined as the number of grams of salt per kilogram of sample (parts per thousand or ppt). The meter was accurate ≥ 0.7 ppt. Temperature was compensated for when determining the salinity. Conductivity in $\mu\text{mhos/cm}$ was also measured, reflecting the identical conductance a sample

would have shown if measured between opposite faces of a 1 cm cube. The conductivity reading was accurate to $\pm 3.0\%$.

CLIMATOLOGICAL STUDIES

Daily climatological data was obtained for the locale from the National Weather Service Office at R.E. Byrd International Airport, a summary of which was published by the National Oceanic and Atmospheric Administration (NOAA). Readings were taken at latitude 37°30' N and longitude 77°20' W at a ground elevation of 164 feet. From these results mean ambient, and maximum and minimum temperatures were considered, as well as precipitation.

BACTERIOLOGICAL STUDIES

The bacterial flora of the lakes being studied was assessed through determinations of MPN (principally Enterobacteriaceae) and fecal coliform counts. MPN's were determined using standard EPA methods outlined in the Standard Methods for the Examination of Water and Waste-water, 14th edition, 1975. Appropriate dilutions of water samples were made and placed in Lauryl Tryptose Broth (Difco 0241-02-7) and if positive, were transferred to indicator tubes containing brilliant green to determine the number of bacteria per 100 cc water.

Identification of bacteria from the lakes being studied was accomplished by adding 10 ml of lake water to a sterile test tube of 10 ml of Lauryl Tryptose Broth (Difco), incubated at 37°C overnight, and inoculated onto eosin methylene blue agar (EMB) (Difco 0076-02-7) and blood agar (BA) consisting of defibrinated sheep blood in a tripticase soy agar base. All Gram negative bacilli were identified using API (ANALYTAB) strips.

In vitro study of the toxicity of some of the bacteria isolated from lakes against pathogenic amebas was undertaken. Nonnutrient agar plates were seeded with a suspension of an 18 hour culture of the bacteria in question. The plates subsequently were inoculated with N. fowleri and incubated at 44°C. Plaque diameters were measured and recorded at 24 and 48 hours. Two strains of Serratia marcescens, received from Dr. R. P. Williams of Baylor College of Medicine were also studied. These strains were mutants of a pigment producing S. marcescens and contained the monopyrrole and dipyrrole non-visible pigments, respectively, but not the visible tripyrrole red pigment (vide infra, SPECIAL STUDIES).

IMMUNOLOGICAL STUDIES

Using a formalinized suspension of N. fowleri, rabbits were inoculated and antisera recovered for agglutination and immunofluorescent studies. The procedure and methods for agglutination were according to that described by Anderson and Jamieson (13). Three strains of N. fowleri were used in this study; W. M., T. Y., and an environmental isolate No. 931. Preparing the antigen for rabbit inoculation involved suspending the ameba in 0.25 percent formal saline to give an approximate concentration of 50,000 trophozoites per ml, determined on a hemocytometer counting chamber. Preimmunizing serum was drawn and two rabbits per strain were immunized by

the intravenous route. Initially 0.25 ml of antigen was injected intravenously. Rabbits were bled by ear vein on day 15 and final bleeding took place on day 21. The antisera were then stored at -70°C for future use in immunofluorescent studies of trophozoites (see APPENDIX A-9).

SPECIAL STUDIES

Studies Conducted by Virginia Polytechnic Institute and State University (VPISU).

The following chemical and biological studies were performed in the Department of Biology and Center for Environmental Studies on samples collected by our laboratory. Those responsible for analysis of the samples were Albert C. Hendricks, Ph.D., Associate Professor of Biology; George Simmons, Ph.D., Associate Professor of Biology; and William H. Yongue, Jr., Ph.D., Associate Professor of Biology.

Samples of water and bottom soil were obtained on March 7 and 8 (winter samples), 1977, at Lakes Overhill, Salisbury, Pocahontas, Falling Creek, Manchester, Pallisades, and Anna I, II, and III; and again on August 23 (summer samples), 1977, at Moore's Lake and Lakes Overhill, Salisbury, Pocahontas, Falling Creek, Manchester, Swift Creek, and Anna I, II, and III.

Water chemistries. Water for chemical and metals analyses was collected in acid-washed test tubes, stored on ice, and taken immediately to the laboratories at VPISU (Methods for Chemical Analysis of Water and Wastes, EPA-625/6-74-003). The metals were determined on a Perkin-Elmer 460 Atomic Absorption Spectrophotometer. Water for organic carbon analysis was collected in acid-washed screw cap vials, stored on ice, and taken to the laboratories and frozen later to be analyzed. Analyses were performed on an Ionics Total Carbon Analyzer. Test for alkalinity, hardness, pH, conductivity, phosphorus, nitrogen, sulfates, and chloride were performed as outlined in Standard Methods for the Examination of Water and Wastewater, 14th Edition, 1975.

Sediment analysis. Bottom soil samples were collected and stored on ice for transport to the laboratories and analyzed according to Methods of Soil Analysis, Part II, Chemical and Microbiological Properties (Edited by C. A. Black and published by American Society of Agronomy, Inc., Madison, Wisconsin, 1965). The samples were then dried at 80°C overnight, ground in a mortar and pestle, and then passed through a number 40-mesh sieve. The portions of sediment which passed through were used for analyses.

Known amounts of dried sediments were heated to 250°C for two hours and then extracted with diluents of H_2SO_4 . A portion of this was utilized for total phosphate ($\text{T-PO}_4\text{P}$) tests, and the remainder was used for metal analyses on the atomic absorption unit. Tests for orthophosphates were performed in the same manner as those for $\text{T-PO}_4\text{-P}$, except the sediments were not heated to 250°C . Known amounts of dried sediments were added to Kjeldahl flasks and the standard Kjeldahl nitrogen tests performed. Ash-free dry weights were determined by weighing the sediments before heating to 550°C and then reweighing. The difference between the two was considered

due to loss of organic carbon.

Biological studies were performed principally by Dr. William Yongue and consisted of evaluating the microbiota present, principally protozoa, and of studying the dynamics of microcolonization of the lakes by protozoa. This was accomplished by placing polyurethane foam units (PF) in each lake as substrates according to the method of Cairns et al (14) and Yongue and Cairns (15) at the same time samples were taken for special chemical analyses (*vide supra*). PF units were submerged and anchored in the epilimnion of Lakes Overhill, Salisbury, Pocahontas, Falling Creek, Manchester, and Anna I. After 14 days of exposure, the PF units were collected, placed in widemouth screw-capped jars containing water from the immediate collecting area and placed on ice for delivery to the laboratories at VPISU. Samplings were completed within 36 hours after having been removed from the ice packing.

In addition, at the same time water was obtained for chemical analyses, samples were obtained by hauling a 10 micron mesh plankton net vertically and horizontally. Plankton net sampling was conducted on the above-mentioned lakes, as well as Anna II and III and Pallisades.

In Vitro Study of Effect of Lake Bacteria Against Pathogenic Amebas

The predominant (>10% frequency of isolation) bacterial species isolated from lakes in this survey were tested for their possible "toxic" and/or inhibitory effect against pathogenic amebas. Nonnutrient agar plates were seeded with suspensions of bacteria grown at 37°C isolated from various lakes. A 9 mm well was then cut into the center of the plate, which was then inoculated with a 0.01 ml suspension of approximately 10^4 N. fowleri (W. M.). Plaque diameters created by the amebas at 44°C were measured at 24, 48, and 72 hours. Plaque diameters were interpreted as reflecting the motility, feeding, growth, multiplication, survival, and encystment of Naegleria (i.e., state of viability). Arithmetic means were calculated, based on experiments.

Further studies focused on the inhibitory effects of bacterial isolates of Serratia (isolated from lake waters) against pathogenic amebas. Those bacteria studied were identified as either Serratia marcescens (three of which were pigmented at room temperature) or Serratia species determined by the API 20 E strip for identification of Enterobacteriaceae). To test these bacteria against amebas, a 9 mm well was cut into the center of a nonnutrient agar plate and partially filled with nonnutrient agar, leaving only a small recess. The plates were then seeded with suspensions of the Serratia in question (all grown at room temperature to allow pigment production), spread evenly over the plate and allowed to dry. Next, a 0.01 ml suspension of approximately 10^4 pathogenic amebas (N. fowleri, T. Y.) were placed into the well. Plaque diameters created at 44°C by the amebas were examined at 24, 48, and 72 hours. Enterobacter cloacae was used as a control and arithmetic means were calculated based on six experiments.

Concomitantly two mutant strains of Serratia marcescens producing the non-visible mono- and bipyrrrole precursors of the visible red pigment, pro-

digiosin (a tripyrrole compound formed by various strains of Serratia) were tested for their inhibitory effect on the growth of pathogenic amebas. Serratia strains WF and 933 (obtained from Dr. R. P. Williams, Baylor University), which produced a volatile monopyrrole and a nonvolatile bipyrrole, respectively, both colorless precursors of prodigiosin, were seeded onto the nonnutrient agar in a manner the same as above. Then a 0.01 ml suspension of approximately 10^4 pathogenic amebas (N. fowleri, T. Y.) were placed onto the centrally located recess well. Plaque diameters at 44°C were measured at 24, 48, and 72 hours.

Study of Effect of Various Antiamebic Agents Against Wild Strains of Pathogenic Amebas Isolated from Lakes in Virginia

During the study, a field isolate of pathogenic N. fowleri strain No. 931 was compared with two human pathogens of N. fowleri (W. M. and T. Y.) for susceptibility to various known antiamebic agents. Strain No. 931 was isolated from Lake Anna I on 10/4/78.

Tissue culture chamber slides were filled with 0.4 ml of each agent in twofold dilutions. To the chambers were added 0.1 ml suspension of 48 hour liquid axenic cultures (CSYECM) of 10^4 of the organisms in question. Amebas were observed after six hours for inhibition of motility, which was represented by rounding-up and loss of pseudopodial activity. Inhibition of growth was observed after 24 and 48 hours. The concentrations of the agent which appeared to effectively inhibit growth was defined as the Minimum Inhibitory Concentration or MIC. The chambers were then emptied of media and refilled with 0.5 ml of Nelson's liquid axenic media without the agent in question. Viable organisms which remained (seen adhering to the chamber walls) grew in Nelson's agent-free media. Chambers were examined on an inverted microscope after 48 hours for growth. The Minimum Amebicidal Concentration (MAC) was defined as that concentration which effectively inhibited viability and multiplication of amebas as recognized by absence of growth within Nelson's media upon subculture.

Agents tested were amphotericin B dissolved in dimethyl sulfoxide at a maximum concentration of five percent (range, 6.25 µg/ml to 0.0098 µg/ml); Amphotericin B methyl ester (range, 25 µg/ml to .098 µg/ml); furamide dissolved in dimethyl sulfoxide at a maximum concentration of 20 percent (range, 25 µg/ml to 0.39 µg/ml); minocycline (range 50 µg/ml to .098 µg/ml); a combination of amphotericin B dissolved in dimethyl sulfoxide (.078 percent for .0975 µg/ml). Also tested were NaCl and dimethyl sulfoxide (DMSO), which used as a solvent for some of the agents.

Study of Effect of Prolonged Incubation of Samples at 44°C on Isolation of Amebas

Bottom soil samples, similar in volume to those obtained during routine collections were processed concomitantly in the usual manner after incubation at 44°C for 24, 100, 200, 300, 400, and 500 hours. Otherwise, samples were processed and subcultured in the routine manner (see APPENDIX A).

A second prolonged incubation study involved a bottom soil sample col-

lected from Lake Anna III (959). Aliquots from the bottom soil sample were removed at designated intervals and processed according to established procedures for isolation and identification of pathogenic amebas. The aliquots of the bottom soil sample were incubated from 24 to 1000 hours (42 days).

Study of Value of Sampling with a Plankton Net in an Attempt to Isolate Pathogenic Amebas

A plankton net (VUR Scientific No. 66175-253) was attached to the rear of a rowboat and pulled for ten minutes through the water. The sediment and particulate matter captured in the net was then transferred in the laboratory to nonnutrient agar plates seeded with *E. aerogenes*. The plates were then incubated at 44°C and examined for seven days thereafter for the presence of amebas.

SECTION 5

RESULTS AND DISCUSSION

ISOLATION OF AMEBAS FROM LAKES

The data contained in this report represents information acquired between December 3, 1976, the time of commencement of sampling, and August 14, 1979. However, the study period itself extended from October 1, 1976 through September 30, 1979.

During the study, seven lakes were sampled continuously, while two lakes, Moore's and Pallisades, were sampled for only two limited periods of time (which was beyond the control of the investigator); namely, from 7/13/77 through 9/15/77 and from 12/20/76 through 5/2/77, respectively. In most instances sampling was biweekly; occasionally for a few lakes and for a limited time it was weekly. During this time, 1,311 samples of soil or water were secured from nine different lakes representing 17 different sampling sites (see TABLE 2).

Included among the 1,311 samples were 645 water samples, each obtained by column filtration of 72 liters of lake water, and 666 bottom soil samples, each obtained by carefully removing approximately 500 gms of the top layer of the surface bottom. In the first year of the study, only shallow water and bottom soil samples were secured; however, after it was learned that investigators in Florida were isolating pathogenic amebas from deep water (Wellings, F. M., personal communication, March, 1977), samples of soil and water also were obtained concomitantly from "deep" sites at Lakes Pocahontas, Salisbury, Falling Creek Reservoir, Moore's, and Swift Creek Reservoir.

Of the 1,311 samples secured, a total of 919 contained free-living amebas or ameboflagellates which survived and multiplied at 44°C after 48 hours of incubation. Morphologically, all of these isolates appeared by the criteria outlined (see MATERIALS AND METHODS, IDENTIFICATION OF AMEBAS) to be small, limax amebas, probably *Acanthamoeba-Hartmannella* or *vahlkampfiids*. No effort was made at this stage to further speciate these isolates, other than to determine that they did or did not flagellate, and if they did flagellate, that they were or were not pathogenic. The assumption was that any small, limax amebas which could be induced to flagellate and form round double-walled cysts belonged to the genus *Naegleria*.

Of the 919 samples which contained small, free-living amebas or ameboflagellates growing at 44°C, only 213 (23.2 percent) were indeed ameboflagellates or presumably *Naegleria*. Thus, it was clear that many small, nonfla-

TABLE 2 . SUMMARY OF ISOLATION OF AMEBOFLAGELLATES (AF) AND
NONFLAGELLATING AMEBAS (NFA) GROWING AT 44°C FROM WATER AND BOTTOM SOIL
OF LAKES IN VIRGINIA (SAMPLING PERIOD: DEC. 3, 1976-AUG. 14, 1979)

Lake	Total No. of Samples Obtained	Total No. of AF Isolated	Total No. of NFA Isolated	Total No. of Water Samples	Total No. of AF From Water	Total No. of NFA From Water	Total No. of Bot. Soil Samples	Total No. of AF From Bot. Soil	Total No. of NFA From Bot. Soil
Overhill	60	3 (5.0%)	38 (63.3%)	30	1 (3.3%)	13 (43.3%)	30	2 (6.7%)	25 (83.3%)
Overhill Feeder	63	9 (14.3%)	43 (68.3%)	32	3 (9.4%)	19 (59.4%)	31	6 (19.4%)	24 (77.4%)
Anna I	130	44 (33.8%)	91 (70.0%)	64	15 (23.4%)	34 (53.1%)	66	29 (43.9%)	57 (86.4%)
Anna II	127	35 (27.6%)	56 (44.1%)	62	12 (19.4%)	27 (43.5%)	65	23 (35.4%)	29 (44.6%)
Anna III	123	12 (9.8%)	77 (62.6%)	60	3 (5.0%)	35 (58.3%)	63	9 (14.3%)	42 (66.7%)
Pocahontas	105	12 (11.4%)	54 (51.4%)	56	6 (10.7%)	23 (41.9%)	49	6 (12.2%)	31 (63.3%)
Pocahontas Deep	68	7 (10.3%)	48 (70.6%)	34	3 (8.8%)	24 (70.6%)	34	4 (11.8%)	24 (70.6%)
Salisbury	94	13 (13.8%)	42 (44.7%)	47	3 (6.4%)	15 (31.9%)	47	10 (21.3%)	27 (57.4%)
Salisbury Deep	62	12 (19.4%)	34 (54.8%)	29	4 (13.8%)	11 (37.9%)	33	8 (24.2%)	23 (69.7%)
Manchester	65	11 (16.9%)	21 (32.3%)	26	5 (19.2%)	7 (26.9%)	39	6 (15.4%)	14 (35.9%)
Falling Creek	117	12 (10.3%)	58 (49.6%)	58	4 (6.9%)	23 (39.7%)	59	8 (13.6%)	35 (59.3%)
Falling Creek Deep	84	8 (9.5%)	46 (54.8%)	42	3 (7.1%)	15 (35.7%)	42	5 (11.9%)	31 (73.8%)

(continued)

TABLE 2 (continued)

Lake	Total No. of Samples Obtained	Total No. of AF Isolated	Total No. of NFA Isolated	Total No. of Water Samples	Total No. of AF From Water	Total No. of NFA From Water	Total No. of Bot. Soil Samples	Total No. AF From Bot. Soil	Total No. NFA From Bot. Soil
Moore's	15	6 (40.0%)	3 (20.0%)	7	1 (14.3%)	1 (14.3%)	8	5 (62.5%)	2 (25.0%)
Moore's Deep	13	7 (53.8%)	2 (15.4%)	6	1 (16.7%)	1 (16.7%)	7	6 (85.7%)	1 (14.3%)
Pallisades	14	1 (7.1%)	5 (35.7%)	7	0 (0.0%)	3 (42.8%)	7	1 (14.3%)	2 (28.6%)
Swift Creek	90	12 (13.3%)	50 (55.6%)	45	4 (8.9%)	19 (42.2%)	45	8 (17.8%)	31 (68.9%)
Swift Creek Deep	81	9 (11.1%)	38 (46.9%)	40	4 (10.0%)	9 (22.5%)	41	5 (12.2%)	29 (70.7%)
Totals	1311	213 (16.2%)	706 (53.9%)	645	72 (11.2%)	279 (43.3%)	666	141 (21.2%)	427 (64.1%)

gellating, free-living, limax amebas other than Naegleria could be found in the waters of many lakes when specimens of soil or water taken from them were incubated at 44°C; and that the selective temperature of 44°C could not be depended upon entirely to select out Naegleria from other free-living amebas that might be present in the samples. Furthermore, in subsequent studies (vide infra, see PATHOGENIC ISOLATES), neither was it demonstrated that all Naegleria growing at 44°C were pathogenic; indeed, only 13 of 213 (6.0 percent) such isolates were pathogenic. Examined from another viewpoint, 76.8 percent of amebas isolated from 919 samples incubated at 44°C were nonnaeglerial, yet potentially pathogenic amebas, if pathogenicity might be suspected by growth at 44-45°C as suggested by Griffin (8). Thus, although the pathogenicity of these nonnaeglerial isolates was never tested, the possibility that they were pathogenic certainly existed.

Analysis of bottom soil yielded more isolates of both amebas and amebflagellates (568 or 43.3 percent of the total isolates) than did analysis of water (351 or 26.7 percent of the total isolates). These differences were even more striking when isolation of only amebflagellates was considered, in which case bottom soil samples were positive in 141 of 666 instances (21.3 percent), while water samples were positive in only 72 of 645 instances (11.2 percent). These differences, which favored soil for isolation of amebflagellates (and thus Naegleria), probably were related to the fact that amebas attached or adhered to the surfaces of soil particles or debris for purposes of feeding (trophozoite stage); or they encysted, in which case in undisturbed water they probably sedimented to the bottom and formed a portion of the top layer of the lake bottom. If a sample volume of lake water larger than 72 L had been filtered, perhaps more samples of water may have been positive than was the case; however, the volume of a water sample necessary to equal the expected recovery of amebflagellates from a bottom soil sample remained unknown, but it probably was considerable. Volumes larger than 72 L with the equipment available would have been time-consuming and impractical to obtain.

Thus, the results of water and bottom soil sampling indicated that large numbers of free-living amebas, and more importantly amebflagellates, could be found in "small" samples (500 gm) of soil more frequently than in large volumes of water (72 L) by the methods employed, and reproducibly so. The results also indicated that in many instances in which water samples were positive for amebflagellates, bottom soil samples obtained concomitantly were also positive (38.6 percent of the total), while on the other hand, 44.6 percent of the 213 samples of bottom soil samples were positive for amebflagellates when concomitant water samples were negative. Only 16.9 percent of the 213 samples positive for amebflagellates represented isolates obtained from water only; i.e., concomitant soil samples were negative. Hence, the technique of securing and culturing bottom soil in search of free-living amebas and amebflagellates appeared to be more sensitive, easier to perform, and more economical than that of examining water; and furthermore, the soil sampling technique appeared to lend itself well to utilization by public health officials or environmentalists for screening fresh-water lakes to assess the pathogenic amebic load and thus the risk of acquiring PAM. Nevertheless, it was clear that for a complete or compre-

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hensive picture, a study or analysis of water as well as soil was necessary.

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In addition, the data suggested that heavily concentrated suspensions of free-living amebas in undisturbed, natural waters occurred infrequently in the lakes under study and probably represented an uncommon natural mechanism or source per se whereby PAM was acquired. This assumption was based on several laboratory experiments utilizing the techniques of water sampling outlined (see APPENDIX A), in which it was found that approximately one trophozoite per 10 ml would have to be present before a 50 percent chance of detection existed. (In such experiments, it was found that the technique of sampling used occasionally detected a concentration of one trophozoite per 100 ml.) Thus, exposure of bathers to high concentrations of pathogenic amebas probably would occur infrequently in undisturbed waters; however, the same might not be said of "muddy" waters.

ISOLATION OF AMEBAS FROM THERMALLY-ENRICHED LAKES

The results of studies of Lakes Anna I, II, and III, the thermally enriched canals and reservoir, respectively, of the Lake Anna system deserve special comment and separate presentation. Although other investigators have isolated pathogenic Naegleria from thermally enriched waters (9,10,16), no one to date has studied carefully a lake or impoundment before, as well during and after, thermal enrichment. Therefore, the relationship of thermal enrichment (or thermal pollution) of fresh-water lakes to the presence or appearance of pathogenic Naegleria has not been correlated. Were pathogenic amebas already present before thermal enrichment occurred, or did they appear after thermal enrichment? If present prior to enrichment, did such enrichment amplify their presence? If amplified, did such waters serve as a nidus for spread of pathogenic organisms to lakes and rivers in other locales? Or, on the other hand, did thermal enrichment exert no selective pressure or have no impact, as suggested but not proven by Wellings in her studies of Florida lakes used for cooling power plants (10)? These questions and more have not been answered; but because the data reported herein were obtained from Lakes Anna I, II and III for more than a year prior to thermal enrichment and for more than a year afterwards, evaluation of the effect(s) of such enrichment on these lakes was possible.

As seen in TABLE 3, ameboflagellates were isolated readily from all three subdivisions of the Lake Anna system throughout 1977 until April, 1978, when the number one reactor of the VEPCO North Anna Power Station was activated for the first time. During this period ameboflagellates were more often isolated from Lake Anna I (12 of 17 months sampled) than from Lake Anna II (five of 17 months sampled) or from Lake Anna III (five of 17 months sampled) and were more often isolated during the hot summer months than during the cool fall, winter or spring ones. However, at no time during this period prior to activation were pathogenic Naegleria recovered from this lake system; yet, during this sampling period, they were isolated from several, nearby, fresh-water lakes under study (L. Pocahontas in June and July and Swift Creek Reservoir in June, 1977), indicating that the detection system was sensitive. Thus, pathogenic ameboflagellates were either not present in the Lake Anna waters prior to activation of the reactor, or they were there in numbers too small to detect by the methods employed.

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TABLE 3. AMEBOFLAGELLATES AND PATHOGENIC AMEBAS ISOLATED
FROM LAKES IN RICHMOND, VIRGINIA AREA
(OCT. 1976-SEPT. 1979)*

Lake	1976	1977												1978												1979												
	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	
Overhill	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Overhill Feeder	0	0	0	0	0	0	0	0	0	0	0	0	-	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Anna I	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+
Anna II	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
Anna III	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pocahontas	-	-	-	-	-	-	-	-	-	-	-	-	P	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pocahontas Deep	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salisbury	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
Salisbury Deep	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Manchester	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Falling Creek	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Falling Creek Deep	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Moore's	0	0	0	0	0	0	0	0	0	0	0	0	+	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Moore's Deep	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Pallisades	-	+	-	-	-	-	-	-	-	-	-	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Swift Creek	-	-	-	-	-	-	-	-	-	-	-	-	P	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Swift Creek Deep	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

*(+) = Ameboflagellates growing at 44°C; (-) = Nonflagellating amebas growing at 44°C; (P) = Pathogen;
(0) = Not tested.
+ = Grew well in Chang's liquid media at 37°C, but growth not sustained.

After the nuclear unit was activated on April 5, 1978, increased numbers of samples became positive for ameboflagellates, especially for nonpathogenic ameboflagellates and amebas which would grow in Chang's liquid CSYECM and, most importantly, for pathogenic Naegleria which were now isolated for the first time. After reactor activation, ameboflagellates were recovered from Lake Anna I, 12 of the 16 months sampled, from Lake Anna II, 13 of the 16 months sampled, and from Lake Anna III, four of the 16 months sampled (see TABLE 3). In addition, ameboflagellates were isolated as frequently during winter months as during summer months, a situation which did not exist prior to reactor activation, and a situation which never existed for the other lakes under study in the area.

The first pathogenic Naegleria isolated after activation of the nuclear unit were in June, 1978, from Lake Anna III, the main reservoir into which the coolant canals and lagoons emptied and a lake used for recreational purposes. In August, 1978, and later in March, 1979, additional pathogenic isolates were recovered from Lake Anna II; and in August, September, and October, 1978, pathogens were isolated for the first time from Lake Anna I, a sampling site located next to the thermal plume (see Figure 5). However, during this post-activation period, only one sample outside the Lake Anna system was positive for pathogenic Naegleria; namely, Falling Creek in August, 1978, suggesting that natural conditions at this time were not necessarily conducive for the appearance of pathogenic Naegleria. A comparison of the Lakes Anna before and after reactor activation (or thermal enrichment) and with other lakes studied in the locale at the same time revealed that of all the physicochemical parameters studied (dissolved oxygen, pH, conductivity, salinity, and temperature), only temperature varied significantly. That is, no noticeably consistent and persistent differences in dissolved oxygen, pH, conductivity, and salinity between Lakes Anna I, II, and III studied before and after activation were noted, nor were any consistent differences between these parameters for the Lakes Anna (I, II, and III) and other lakes under study observed. However, after activation, the temperatures of the waters, especially for Lakes Anna I and II, were consistently higher (see TABLES B-2, B-3, and B-4) than those recorded in the months prior to activation for this system, as well as after activation than for other natural fresh-water lakes in the locale which were studied at the same time. Similarly, no noticeable differences in coliform counts (MPN's) were detected before and after reactor activation and thermal enrichment (see TABLES B-10, B-11, and B-12 in APPENDIX B); however, the data is less complete for this microbiological parameter than for the physicochemical parameters studied; i.e., the presence of absence of various bacterial species was not thoroughly assessed.

It is noteworthy that pathogenic ameboflagellates were isolated less frequently during 1979 than during 1978 from the Lake Anna system, especially during the summer months when none were recovered from Lake Anna I, II, or III (see TABLE 3). This was most likely because water temperatures for these lakes were several degrees higher during the summer of 1978 (24.5°C to 34.8°C) than during the summer of 1979 (24.1°C to 31.2°C) (see TABLES B-2, B-3, and B-4 in APPENDIX B) because of inactivation of the first unit during April-May, 1979, and <100% inactivation of the second unit. Alternative explanations are that some unidentified factors adversely affecting pathogenic Nae-

gleria appeared, e.g., predators, competitors, lack of nutrients, etc. . .

PATHOGENIC ISOLATES

During the study, 59 isolates were examined for pathogenicity by mouse virulence tests (see APPENDIX A-8); 13 were found to be pathogenic. Thus, 13 of 1,311 (one percent) samples secured contained N. fowleri (TABLE B-1 in APPENDIX B). Of these 13 positive samples, five were water and eight were bottom soil. All water and soil samples positive for pathogens were obtained from shallow areas; i.e., of 1,003 samples of water and bottom soil secured from shallow areas only, 1.29 percent were positive for pathogens; and of shallow bottom soil samples only, 1.57 percent (eight of 509 samples) were positive for pathogens. Contrary to the findings of Wellings (10), no pathogens were found in deep water areas, despite two years of sampling, representing 308 deep samples (151 water and 157 soil) from five different lakes: Pocahontas (68 samples), Salisbury (62 samples), Falling Creek (84 samples), Moore's (13 samples) and Swift Creek Reservoir (81 samples).

The reason for the low number and low frequency of isolation of pathogenic Naegleria from Richmond area lakes and the complete absence of such pathogens from deep water areas was not evident. Samples of soil and strains of amebas (both pathogenic and nonpathogenic) were exchanged on several occasions between the Richmond laboratory and that of Dr. Wellings's in Florida for quality control purposes and identical results were noted. Thus, faulty techniques did not appear responsible for these findings. The explanation was probably that pathogenic Naegleria were present in the Richmond lakes in small numbers and did not dominate or occupy a sizeable part of the protozoan community. Florida, being somewhat tropical, and Virginia, located in the temperate zone, represented obviously different situations.

Pathogenic Naegleria were isolated from four separate lakes or impoundments: Lake Pocahontas, Swift Creek Reservoir, Falling Creek Reservoir, and Lake Anna, and from all three sampling sites in the Lake Anna system; i.e., Lakes Anna I, II, and III (TABLE 3). For Lake Anna I, four of 130 (3.1 percent) samples taken were positive for pathogenic Naegleria; and for Lakes Anna II and III, two of 127 (1.6 percent) and two of 123 (1.6 percent), respectively, were positive. Moore's Lake, a lake previously suspected of being the source for several cases of PAM, yielded no pathogenic Naegleria from a total of 28 samples (15 shallow and 13 deep); however, considering the expected low frequency of isolation of pathogenic Naegleria in the locale, the sample size was inadequate to have statistical meaning. (Based on an expected frequency of three percent for isolation of pathogenic Naegleria, the number of samples from Moore's would have had to have been 98 for a 95 percent certainty that enough samples were tested to draw meaningful conclusions (17).) In only one instance was a concomitant water and bottom soil sample shown to contain pathogenic Naegleria (Swift Creek Reservoir on June 27, 1977). Often, when pathogenic Naegleria did appear, they did so in clusters; i.e., several isolates were obtained during the same month or time frame (see TABLE 3 and TABLE B-1 in APPENDIX B).

Pathogens isolated in June and July, 1977 (strain numbers 223, 234, 236, and 299) and in June, 1978 (strain number 742) were of low virulence; i.e.,

less than 50 percent of the mice inoculated intranasally died. In several instances the presence of amebas in the brains of those mice which died was overlooked because small numbers of amebas were present; only after subculture were they detected. In addition, the usual severe hemorrhagic necrosis of the brain was not noted. Eventually these isolates were lost, despite repeated attempts to sustain them in Chang's liquid CSYECM and in mice. Perhaps they represented intermediate pathogens; i.e., organisms between "N. fowleri-immunopositive-but-nonpathogenic" and "N. fowleri-immunopositive-pathogenic" Naegleria or organisms of low virulence similar to N. jadini isolated from a fresh-water stream in Belgium (18) or strain PP397 isolated by Jamieson in 1973 (19).

The seven pathogens isolated in 1978, all but one of which were isolated from the Lake Anna system, were highly pathogenic for mice and highly virulent. For isolate #858 (from bottom soil of Lake Anna I), 100% of mice (ten of ten) were killed by as few as 1.1×10^4 trophozoites instilled intranasally. Similarly, the one positive isolate (#1122 from the bottom soil of Lake Anna II) obtained in 1979 killed 100 percent (five of five) mice with 0.9×10^4 trophozoites instilled intranasally. All of these pathogens isolated in 1978 and 1979 were easily cultured and easily sustained in Chang's liquid axenic media (CSYECM). In addition all have maintained their pathogenicity to date, despite multiple passages and stocking.

During the study, 13 nonflagellating limax amebas which grew at 44°C were isolated from 13 samples (12 of which were bottom soil) and were tested for pathogenicity. This was done because after the study commenced, it was decided to inoculate an agar plug from every amebic plaque with sharp lines appearing after 24-48 hours at 44-45°C incubation, while at the same time subjecting them to flagellate induction. Twelve of these isolates were found to grow well in Chang's liquid CSYECM initially and were directly instilled intranasally into mice; however, none of the isolates proved to be pathogenic for mice and none could subsequently be sustained in Chang's liquid axenic media.

The majority of pathogenic Naegleria were isolated from samples obtained from lake water or bottom soil when water temperatures were elevated; i.e., above 30°C (vide infra, PHYSICOCHEMICAL STUDIES and vide supra ISOLATION OF AMEBAS FROM LAKES), particularly if the temperature elevation was sustained for a prolonged period of time. Ten of 13 pathogenic isolates were obtained when the water temperature was >30°C; seven of 13 were obtained when the water temperature was >33°C; and four of 13 when it was >35°C (see TABLE 5). Examined from another point of view, the percent of ameboflagellates appearing in samples began to increase noticeably at 26-30°C; and the percent of pathogenic ameboflagellates increased at 33°C (18.8 percent at 33-34°C as compared to 3.8 percent at 31-32°C). Thus, sustained elevated lake water temperatures played an important role in the appearance of pathogenic forms of free-living amebas, especially when those temperatures approximated 30°C continually for one to two months.

PHYSICOCHEMICAL STUDIES

TABLE 4. MEAN PHYSICOCHEMICAL CHARACTERISTICS
OF NINE LAKES SAMPLED IN THE RICHMOND, VIRGINIA AREA OVER A THREE
YEAR PERIOD (DEC. 3, 1976-AUGUST 14, 1979)

Lake	Site No.	Temp. °C	pH	Dissolved O ₂ PPM	Salinity ppt	Conductivity µmhos/cm
Overhill	1	15.5	7.4	9.9	0.2	879.0
Overhill Feeder	14	11.4	5.5	7.7	0.0	107.0
Anna I	2	17.0	6.6	9.1	0.1	152.8
Anna II	3	16.2	6.7	9.2	0.1	181.5
Anna III	4	14.8	6.7	9.3	0.0	108.8
Pocahontas	5	17.8	6.3	9.3	0.0	55.9
Pocahontas Deep	18	18.6	6.4	6.2	0.0	95.7
Salisbury	6	17.8	6.0	9.4	0.0	34.0
Salisbury Deep	13	18.1	5.8	8.8	0.0	38.8
Manchester	7	18.2	6.8	10.4	0.0	112.8
Falling Creek	8	15.4	6.5	9.8	0.0	66.3
Falling Creek Deep	17	10.7	6.1	0.6	0.0	90.2
Moore's*	9	27.3	8.3	8.3	0.6	1671.4
Moore's Deep*	19	28.4	8.1	8.3	0.5	1750.0
Pallisades†	10	11.1	6.5	11.6	0.0	49.7
Swift Creek	11	17.6	6.6	9.6	0.0	46.4
Swift Creek Deep	12	16.8	6.5	8.3	0.0	76.6

* Sampled only from 7/13/77 through 9/15/77.

† Sampled only from 12/20/76 through 5/2/77.

A summary of the mean physicochemical characteristics, including temperature, pH, dissolved oxygen (D.O.), salinity, and conductivity is presented in TABLE 4. Each parameter is separately considered below.

Temperature. Throughout the three-year study period the temperature of the lakes examined were reasonably similar, with the exception of Lakes Anna I, II, and III, in which thermal enrichment occurred beginning in April, 1978 (see TABLES B-2, B-3, and B-4 in APPENDIX B). The small differences recorded between natural lakes were probably due to differences in the time when measurements were made, since obviously for logistical reasons every lake could not be sampled exactly at the same time. Striking differences in mean temperatures (TABLE 4) reflected a bias in sampling and in calculations; e.g., Moore's Lake was studied only in the summer of 1977, and the high mean values (27.3°C and 28.4°C) included reflected a bias.

As expected, deep water temperatures were consistently lower than shallow water ones. This was not necessarily reflected by the summary of mean temperatures (TABLE 4), since these calculations included more winter measurements made for shallow sites than for deep ones (since deep sampling was not commenced until the summer of 1977). However, comparison of monthly means for each lake (TABLES B-2 through B-4) reflected these differences more accurately. For some lakes, for example Falling Creek, differences between deep and shallow water temperatures (ΔT°) were greater than those for other lakes. The greater ΔT° 's were probably due to the deeper water (18 feet) sampled for Falling Creek than for other lakes; e.g., Salisbury (six feet), Swift Creek (eight feet), Pocahontas (seven feet), and Moore's (eight feet). In the summer, the ΔT 's for Falling Creek were greater than in the winter, often being as high as 10-15°C; however, for the other lakes subjected to deep and shallow sampling, ΔT 's frequently did not exceed 1 or 2°C.

In general, the number or percent of ameboflagellates correlated with elevated mean water temperatures during the sampling period (Figure 17). As the water temperature rose in the warm months, the percent isolation of ameboflagellates rose; and lakes with consistently high temperatures usually contained a higher percent of ameboflagellates than those with low temperatures. An exception to this generality was Lake Overhill; however, a number and variety of chemicals and germicides (notably chlorine) frequently were added to the water of this lake which might explain the low rate of isolation of ameboflagellates from it. That this might be real was suggested by the fact that the parent lake (Overhill Feeder Lake) which fed water to Lake Overhill, being located adjacent to it but was not subjected to chemical treatment, yielded a rate of isolation of ameboflagellates twice that of its daughter lake.

Similarly, the number and percent isolation of pathogenic Naegleria also appeared to correlate with elevated temperatures, but not invariably so. Ten of 13 (77 percent) of the pathogens (see TABLE 5) were recovered from lake sites in which the water temperatures at the time of sampling were 30°C or higher. Perhaps as important as an elevated water temperature was a sustained elevation of water temperature (Figure 18). It appeared that an elevated water temperature per se; e.g., 30°C, was insufficient to

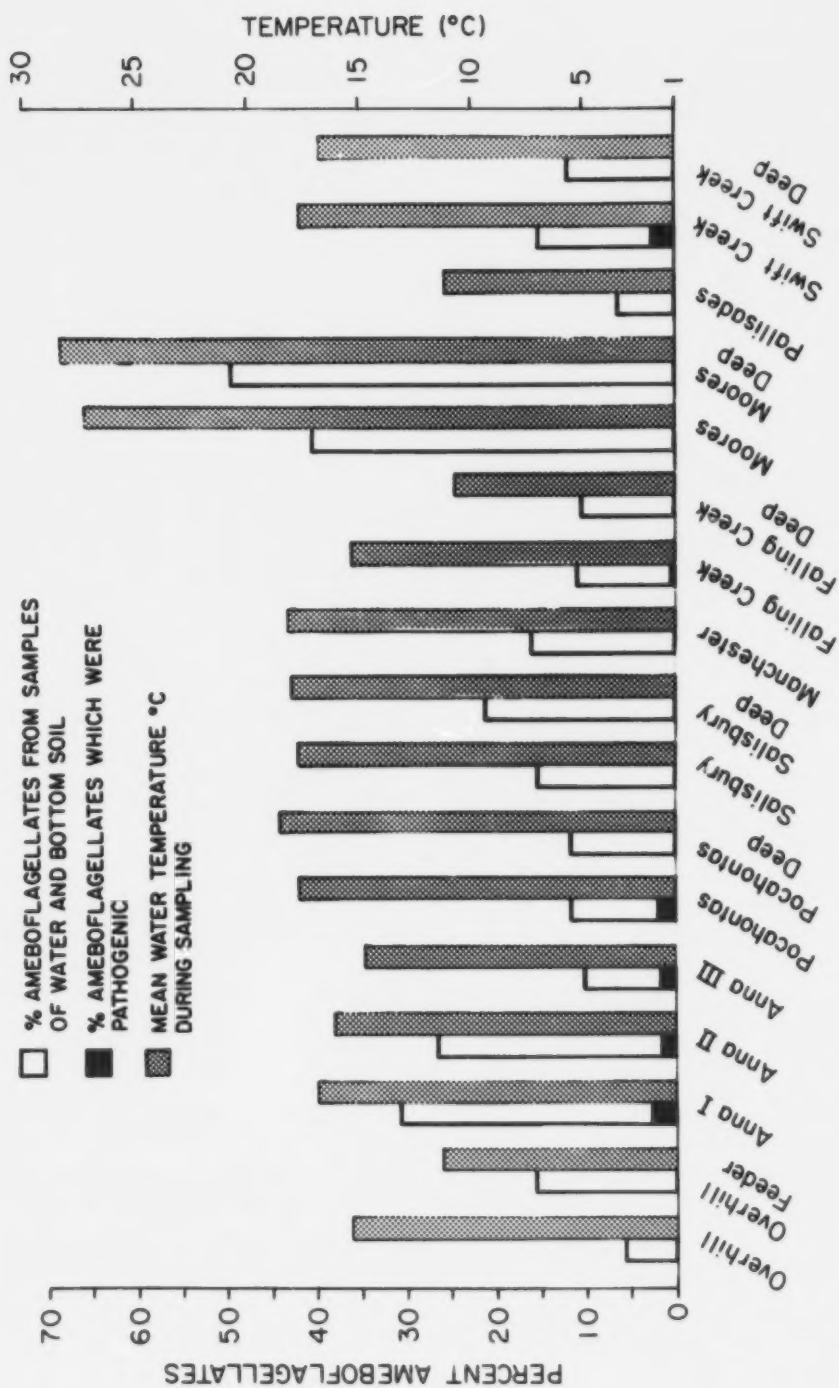


Figure 17. Correlation of mean water temperatures ($^{\circ}\text{C}$) during sampling with recovery of ameboflagellates and pathogenic *Naegleria* for nine lakes studied in Virginia (December 3, 1976 through August 14, 1979).

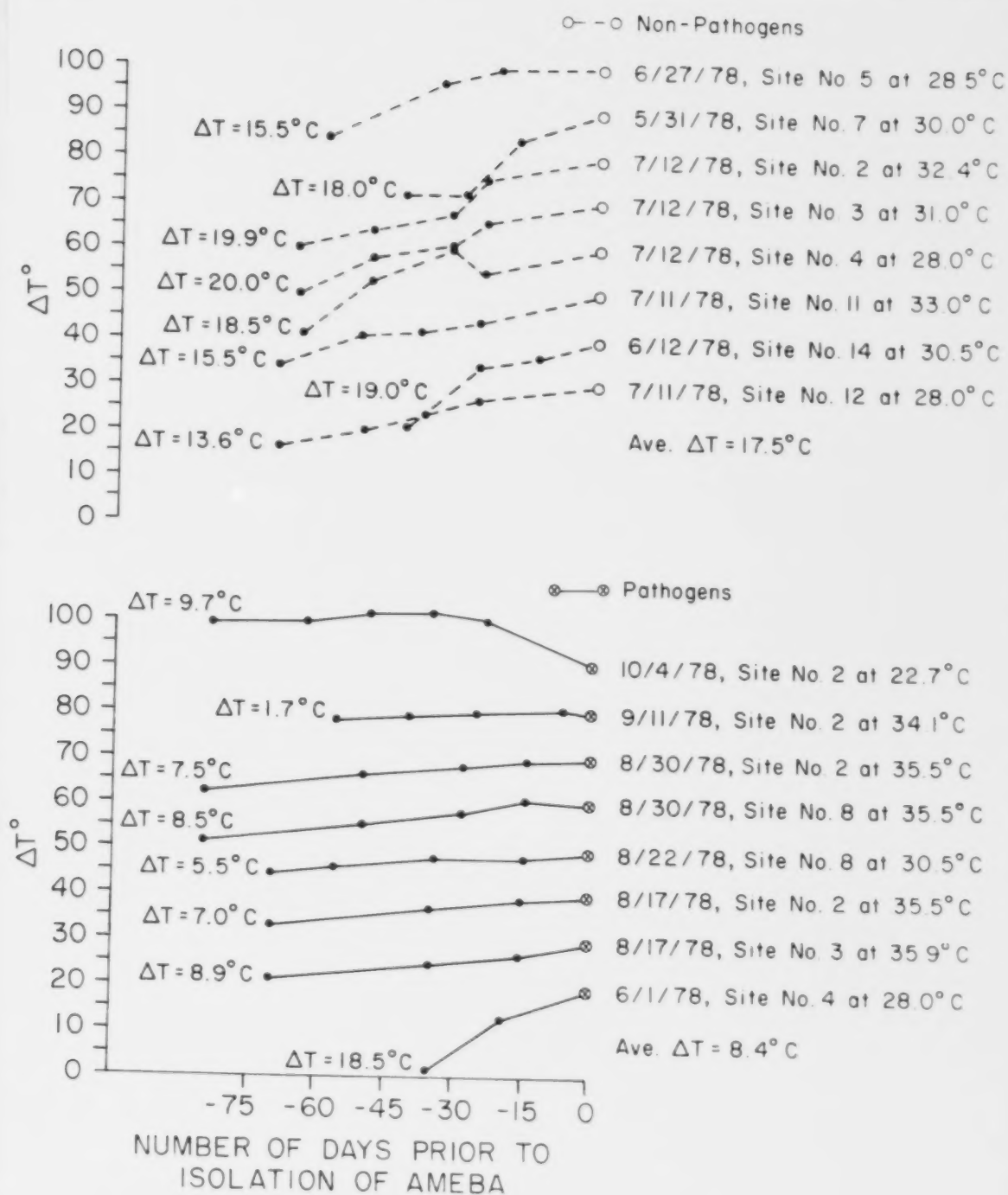


Figure 18. Comparison of ΔT 's associated with isolation of pathogenic and non-pathogenic amebas for several selected sites under study.

precipitate pathogenic forms. However, if the elevation was sustained for a time period of one to two months (that is, the ΔT over this period remained small), then pathogenic forms were likely to appear. The precise time period over which the elevated temperatures must have been sustained was not determined; however, it probably varied for each lake and for different conditions.

It was noted also that the frequency of isolation of ameboflagellates and pathogenic *Naegleria* from the three sampling sites of Lake Anna (Lake Anna I, II, and III) fell proportionately as the temperature gradient between the lake sites declined (*vide supra*, PATHOGENIC ISOLATES).

TABLE 5. NUMBER AND PERCENT OF AMEBOFLAGELLATES AND PATHOGENS (*NAEGLERIA*) ISOLATED AT DIFFERENT LAKE WATER TEMPERATURES (SAMPLING PERIOD DEC. 3, 1976-AUG. 14, 1979)

Water Temp. (°C)	Ameboflagellates*	Pathogens (<i>Naegleria</i>)†
<0-5	9.81% (21/214)	0.0% (0/21)
6-10	9.65% (11/114)	0.0% (0/11)
11-15	10.27% (23/224)	4.3% (1/23)
16-20	7.95% (14/176)	0.0% (0/14)
21-25	11.74% (27/230)	3.7% (1/27)
26-30	27.83% (64/230)	4.7% (3/64)
31-32	52.0% (26/50)	3.8% (1/26)
33-34	61.54% (16/26)	18.8% (3/16)
35-36	78.57% (11/14)	36.4% (4/11)

* Numbers in parentheses represent number of ameboflagellates isolated at given temperature/total number of samples obtained at the given temperature.

† Numbers in parentheses represent number of pathogenic amebas isolated at given temperature/number of ameboflagellates isolated at given temperature.

Elevated water temperatures were associated with the isolation of environmental strains of pathogenic *Naegleria* in a number of recent studies (9, 10, 16, 20, 21, and 21). In 1973, De Jonckheere et al (9) isolated pathogenic *Naegleria* from a thermally-polluted canal in Antwerp, Belgium. The canal

was the origin of a stream which had recently been the source of a fatal case of PAM, and it also supplied the water for several nearby swimming pools which five years before in 1970-72 had been responsible for an epidemic of PAM. The same canal received warm effluents from a lead and zinc factory; producing a water temperature at the thermal plume of 29 to 30°C. More pathogenic Naegleria were isolated from the thermal plume and its immediate surroundings than further away where water temperatures were 22 to 23°C. In addition, isolates of pathogenic Naegleria were more frequently obtained in the summer when the water was at its warmest than in the late fall. However, that elevated water temperatures were not solely responsible for the presence of pathogenic Naegleria was suggested by the fact that no such organisms could be isolated from the adjacent warm waters (29°C) of an electricity power plant at the same water temperature as the lead and zinc factory.

In 1976 Willaert and Stevens (16) reported detecting pathogenic Naegleria in the thermal-discharge water (32-34°C) of electric power stations in Florida, while four nearby control lakes were negative for pathogens. About this same time, Wellings *et al.* (21) reported that the presence of pathogenic Naegleria in Florida lakes was widespread and certainly not limited to power plant discharges; i.e., 12 of 26 lakes sampled only once yielded pathogenic Naegleria, at times reaching levels of one ameba per 25 ml of water during the summer months. Such isolates were generally obtained while water temperatures were 26°C or above, and the number of positive samples reflected the water temperature rise in fresh-water lakes. In August, when water temperatures reached 34.5°C, isolation rates of pathogenic Naegleria were maximal.

Cerva in 1977 (22) examined cooling waters that earlier had been the source of a fatal case of PAM from a coal processing plant in Northern Bohemia. Pathogenic Naegleria were isolated from biological material scrapings and bottom soil when water temperatures were 27 to 30°C.

Stevens *et al.* (20) in 1977 reported that examinations of a number of water samples obtained from waters contaminated by discharges from power plants were positive for pathogenic Naegleria in two of five lakes and rivers in Florida and in one of eight man-made lakes in Texas. The temperatures at the sampling sites were 38°C at Lake Monroe, Florida; 34-35°C at St. John's River, Jacksonville (Southside), Florida; and 39-41° at Lake Arlington, Texas. However, like De Jonckheere *et al.* (9), Stevens *et al.* concluded that other factors beside elevated water temperatures may have contributed to the distribution of pathogenic Naegleria in the environment, since such organisms could not be found in all fresh waters with elevated temperatures.

In summary, from the studies reported herein and from the work of others, it was obvious that water temperature was a central factor in determining whether or not Naegleria pathogenic for man were present or might soon be detected in various lakes. When water temperatures reached or exceeded 30°C, pathogenic Naegleria was likely; however, lower temperatures by no means excluded this possibility. In addition, when such elevated water temperatures were sustained over a period of one to two months, the

likelihood that pathogenic Naegleria would appear in numbers which could be detected was still further enhanced.

pH. The pH of the lakes studied were generally in the range of 6.0 to 6.7 and showed little variation from sample to sample in the same lake and from lake to lake with the exception that Lake Overhill and Moore's Lake were consistently higher than other lakes by one or two pH units (TABLE 4). This was probably because the owners of the two lakes added sodium bicarbonate and other chemicals daily in an attempt to keep bacterial counts down and to clear the water of suspended particles. This was very evident at Lake Overhill where the mean pH for the entire period was 7.4, while the adjacent, undisturbed, natural feeder lake was 5.5.

Free-living amebas of the genera Acanthamoeba and Naegleria are known to do well at pH's between six and seven, and pathogenic Naegleria, especially at seven. Lake Anna and Lake Manchester (with which a case of PAM was associated) all had relatively high pH's; i.e., above 6.5, as compared to other lakes studied which (except Lake Overhill and Moore's) were at a pH of 6.5 or below.

Dissolved Oxygen (D.O.). In general, D.O. when measured at shallow lake water sites at the same time of the year varied little from lake to lake (TABLES B-5, B-6, and B-7. See APPENDIX B). D.O. varied inversely with water temperature. It was highest in the winter and lowest in the summer. Greatest variations between lakes and between consecutive samples obtained from the same lake occurred during the spring, when ambient and water temperatures were fluctuating considerably. Clearly, D.O. did not appear to be the critical factor in controlling the appearance of either ameboflagellates or pathogenic forms; and a direct correlation between D.O. and these parameters was not obvious (Figure 19). However, since D.O. varied indirectly with water temperature, and since isolation of ameboflagellates and pathogens appeared to vary directly with temperature, a relationship between D.O. and the presence of ameboflagellates and/or pathogenic forms probably existed. For example, D.O. was generally lower throughout the three-year study period for the warmer Lake Anna I than for the cooler Lake Anna III, and the former lake site yielded more pathogens than the latter. Perhaps the methods of measuring D.O. were too crude or were associated with too many undetected errors to define such a relationship; or too many other natural factors not examined influenced the final values; so that no clear, consistent correlation could be detected.

D.O., when measured at deep sites, was consistently low and often was too small to measure by the techniques employed. However, ameboflagellates (but not pathogens) were still isolated from these areas, despite the known aerobic nature of these organisms and the expected anaerobic environment of the lake bottoms. Wellings noted similar findings; i.e., that Naegleria could survive and grow under natural conditions under what appeared to be surprisingly anaerobic conditions (10). Most likely, survival was in the cyst form rather than trophozoite; and these forms may have constituted the majority of the population present in the bottom soil of deep areas. However, the methods utilized to isolate Naegleria precluded proof of this hypothesis.

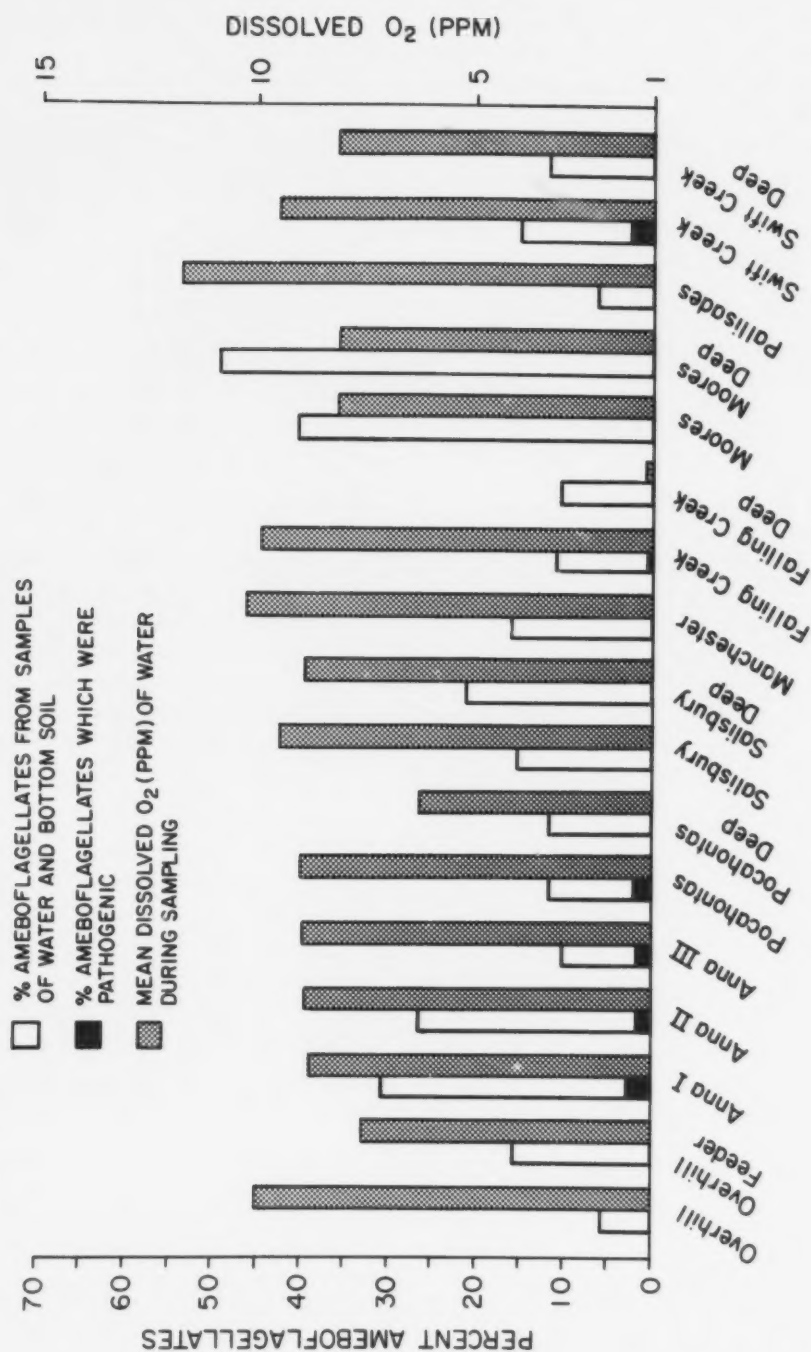


Figure 19. Correlation of mean dissolved oxygen (ppm) of water during sampling with recovery of ameboflagellates and pathogenic *Naegleria* for nine lakes studied in Virginia (December 3, 1976 through August 14, 1979).

Salinity and Conductivity. By the methods of measurement employed, the water of most lakes or lake sites sampled did not contain significant quantities of NaCl. This was not surprising in view of the fact that the lakes under study were 100 miles inland from the Atlantic Ocean and were clearly fresh water. Measurements of NaCl were important to obtain in that pathogenic Naegleria have been noted to die with concentrations of NaCl as little as 0.5 percent. The highest salinity measured in this study was noted with Moore's Lake and Lake Overhill, where large quantities of chlorine, aluminum sulfate and potassium carbonate were being added in unknown quantities daily. This fact was also reflected in measurements of conductivity, in which these two lakes also were highest.

CLIMATOLOGICAL STUDIES

A summary of the climatological changes that occurred during the two-year study period is presented in TABLE 6.

In general, the spectrum of ambient temperatures was similar from year to year; however, the winter of 1978 was extremely harsh (mean temperature for January and February below freezing or -0.15°C), and low temperatures occurred consistently day after day. Nevertheless, the largest number of pathogens were isolated in the summer following this winter, but principally from thermally-enriched lakes (Anna I, II, and III) rather than from natural ones. Since the Lake Anna system was not thermally enriched until the spring of 1978, the data suggested that freezing or below freezing ambient temperatures sustained for two or more months did not destroy pathogenic forms or prevent subsequent blooms when warmer weather followed.

Precipitation, which might reflect run off and thus soil contamination of water, was not significantly different from year to year with the exception of greater precipitation in the winter of 1978 and 1979 as compared to that of 1977. The significance of this, if any, was unclear.

BACTERIOLOGICAL STUDIES

A summary of the quantitative (MPN) and qualitative aspects of the bacterial coliforms (and thus Enterobacteriaceae) found in the various lakes under study is presented in TABLE 7. In addition, a detailed analysis of each lake or lake site is presented in TABLES B-8 through B-21 in APPENDIX B (Moore's and Pallisades Lakes were not studied in this regard because they had been drained or closed by the owners).

The highest mean number of total coliforms were found in Lake Salisbury; however, Overhill Feeder Lake, Lake Anna II, Lake Pocahontas, Lake Manchester and Falling Creek Reservoir were almost as high. Overhill Lake had the lowest mean number of total coliforms (mean MPN was 70 with range of 3 to 430), probably because this lake had many antibacterial chemicals, most notably chlorine, added to it almost daily. No discernible correlation was noted between total coliforms and the presence of ameboflagellates which would grow at 44°C and/or pathogenic Naegleria (Figure 20). This lack of correlation was especially noted for Lakes Anna I, II, and III; i.e., Lake Anna I was associated with the highest frequency of pathogens isolated and

TABLE 6. SUMMARY OF MONTHLY MEAN AMBIENT TEMPERATURE AND RAINFALL IN THE RICHMOND AREA OVER A THREE YEAR PERIOD (OCT. 1, 1976 - SEPT. 30, 1979).

Month	1976-mean		1977-mean		1978-mean		1979-mean	
	Amb. T°C	Rainfallcm	Amb. T°C	Rainfallcm	Amb. T°C	Rainfallcm	Amb. T°C	Rainfallcm
Jan.	-	-	-3.72	5.64	0.77	20.24	2.4	15.65
Feb.	-	-	4.72	3.40	-0.94	1.22	-1.9	15.16
Mar.	-	-	12.06	6.78	6.9	14.4	10.89	6.58
Apr.	-	-	16.17	5.92	14.0	10.9	14.67	10.08
May	-	-	20.11	10.13	18.6	9.9	19.50	9.65
June	-	-	20.78	3.18	23.72	13.36	21.56	6.15
July	-	-	27.44	10.67	25.28	10.77	24.94	11.07
Aug.	-	-	26.56	15.62	26.72	15.06	25.44	17.98
Sept.	-	-	23.44	5.49	22.7	0.66	21.67	24.79
Oct.	12.44	17.75	14.06	20.02	14.6	3.07	NA*	NA
Nov.	5.94	4.78	11.28	10.97	11.4	11.61	NA	NA
Dec.	2.61	6.50	4.17	14.15	5.8	9.65	NA	NA

* NA = not available.

TABLE 7. SUMMARY OF BACTERIAL FLORA OF
LAKES STUDIED IN RICHMOND, VIRGINIA AREA

Lake	Site #	Total Coliform MPN Mean/No. Samples (Range)*	Fecal Coliform MPN Mean/No. Samples (Range)*	Most Predominant Gram Negative Bacilli†
Overhill	1	70/8 (3-430)	15/8 (3-93)	1. <u>Pseudomonas fluorescens</u> 2. <u>Pseudomonas species</u> 3. <u>Serratia marcescens</u> 1. <u>Enterobacter cloacae</u> 2. <u>Escherichia coli</u>
Overhill Feeder Anna I	14 2	4070/8 (10-24,000) 223/8 (3-930)	337/8 (3-2400) 134/9 (3-1100)	1. <u>Klebsiella pneumoniae</u> 2. <u>Pseudomonas species</u> 3. <u>Citrobacter freundii</u> 1. <u>Enterobacter cloacae</u> 2. <u>Escherichia coli</u> 3. <u>Proteus morganii</u> 4. <u>Serratia rubridae</u> 1. <u>Enterobacter cloacae</u> 2. <u>Enterobacter hafniae</u> 3. <u>Klebsiella pneumoniae</u> 4. <u>Serratia marcescens</u> 5. <u>Pseudomonas fluorescens</u>
Anna II	3	5061/10 (3-24,000)	18/9 (3-93)	1. <u>Escherichia coli</u> 2. <u>Enterobacter cloacae</u> 3. <u>Proteus morganii</u> 4. <u>Serratia rubridae</u> 1. <u>Enterobacter cloacae</u> 2. <u>Enterobacter hafniae</u> 3. <u>Klebsiella pneumoniae</u> 4. <u>Serratia marcescens</u> 5. <u>Pseudomonas fluorescens</u>
Anna III	4	207/9 (3-750)	33/9 (3-90)	1. <u>Escherichia coli</u> 2. <u>Enterobacter cloacae</u> 3. <u>Klebsiella pneumoniae</u> 4. <u>Serratia marcescens</u> 5. <u>Pseudomonas fluorescens</u>
Pocahontas	5	(4737/13 (21-24,000)	343/12 (15-1500)	1. <u>Escherichia coli</u> 2. <u>Enterobacter cloacae</u> 3. <u>Klebsiella pneumoniae</u> 4. <u>Serratia marcescens</u> 5. <u>Citrobacter freundii</u>
Pocahontas Deep Salisbury	18 6	2151/10 (130-11,000) 5545/15 (15-24,000)	658/10 (43-4600) 234/15 (3-1500)	1. <u>Klebsiella pneumoniae</u> 2. <u>Enterobacter cloacae</u> 1. <u>Escherichia coli</u> 2. <u>Enterobacter cloacae</u> 3. <u>Pseudomonas fluorescens</u>

(continued)

TABLE 7 (continued)

Lake	Site #	Total Coliform MPN Mean/No. Samples (Range)*	Fecal Coliform MPN Mean/No. Samples (Range)*	Most Predominant Gram Negative Bacilli†
Salisbury	13	4324/14 (9-24,000)	515/14 (3-2400)	4. <u>Proteus morganii</u> 1. <u>Serratia marcescens</u> 2. <u>Escherichia coli</u> 3. <u>Pseudomonas fluorescens</u> 4. <u>Enterobacter cloacae</u> 5. <u>Enterobacter aerogenes</u> 6. <u>Proteus morganii</u> 1. <u>Escherichia coli</u> 2. <u>Enterobacter cloacae</u> 3. <u>Klebsiella pneumoniae</u> 4. <u>Pseudomonas fluorescens</u>
Manchester	7	4016/14 (23-24,000)	745/13 (3-4600)	
Falling Creek	8	4511/14 (23-24,000)	349/14 (3-2400)	1. <u>Escherichia coli</u> 2. <u>Enterobacter cloacae</u> 3. <u>Klebsiella pneumoniae</u> 4. <u>Enterobacter agglomerans</u> 5. <u>Serratia liquifaciens</u> 6. <u>Serratia species</u> 7. <u>Pseudomonas aeruginosa</u> 1. <u>Escherichia coli</u> 2. <u>Enterobacter cloacae</u> 3. <u>Pseudomonas fluorescens</u> N.D.†
Falling Creek Deep	17	1360/11 (16-4600)	42/11 (3-238)	
Moore's	9	N.D.†	N.D.†	N.D.†
Moore's	19	N.D.†	N.D.†	N.D.†
Deep	10	N.D.†	N.D.†	
Pallisades	11	1319/13 (3-7500)	9-13 (3-23)	1. <u>Enterobacter cloacae</u> 2. <u>Enterobacter cloacae</u> 3. <u>Citrobacter freundii</u> 4. <u>Serratia marcescens</u>
Swift Creek				

TABLE 7 (continued)

Lake	Site #	Total Coliform MPN Mean/No. Samples (Range)*	Fecal Coliform MPN Mean/No. Samples (Range)*	Most Predominant Gram Negative Bacilli†
Swift Creek Deep	12	310/11 (3-1500)	9/11 (3-43)	1. <u>Escherichia coli</u> 2. <u>Serratia marcescens</u> 3. <u>Pseudomonas fluorescens</u> 4. <u>Klebsiella pneumoniae</u> 5. <u>Aeromonas species</u>

* Numerator represents arithmetic mean; denominator represents number of tests performed; numbers in parentheses represent range, maximum and minimum.

† Number preceding species name indicates order of predominance on subcultures, i.e., "1" most predominant, "2" next most predominant, etc. . .

‡ N.D. = Not Done.

Lake Anna III the lowest, yet Anna II had the highest mean total coliform MPN, while Lakes Anna I and III had about the same MPN's. Hence, no correlation between the frequency of isolation of pathogens and the concentration (MPN's) of coliforms was apparent.

The same was true for fecal coliforms, which appeared to be highest for Lake Manchester (mean MPN 745), a lake surrounded by residential sites, but which was not associated with the presence of pathogenic Naegleria.

Generally, E. coli and members of the tribe Klebsiella-Enterobacter-Serratia were found in fresh-water samples. Pseudomonas (a non-Enterobacteriaceae) was unusual, but it (notably Pseudomonas fluorescens) was the most frequently found bacterium in Lake Overhill (TABLE B-1), perhaps because this bacterium was selected for by the addition of chlorine and other chemicals.

Some investigators (9,10) have suggested that the appearance of pathogenic Naegleria may depend upon the number of bacteria, especially coliforms, present in the water and available as a food source for amebas; and that the population of pathogenic Naegleria may increase as coliforms increase. This was noted especially in some of the lakes in Georgia studied by Wellings (10).

Klebsiella-Enterobacter-Serratia have classically been an excellent food source for free-living amebas, especially for Naegleria, while Pseudomonas species, particularly those producing pigments, have been recognized as frequently toxic for free-living amebas (23). In subsequent studies (vide infra; see SPECIAL STUDIES, In vitro Study of Effect of Lake Bacteria Against Pathogenic Amebas), these observations were confirmed in vitro and extended. Although quantitatively no significant relationship appeared to exist between the presence of ameboflagellates and/or pathogenic Naegleria and the total numbers of bacterial coliforms, qualitatively there may well have been a relationship, as certain bacteria served as good food sources for amebas, while others (notably Pseudomonas and Serratia) did not and in fact may have inhibited Naegleria. Thus, an inverse quantitative relationship may have existed between the number of toxic bacteria present and the number of Naegleria isolated; however, the study was not designed to answer this question. It was interesting that with the exception of Lake Anna III, pathogenic Naegleria were not isolated from any lake or lake site from which Pseudomonas fluorescens was concomitantly isolated; i.e., Lake Overhill, Lake Salisbury, Lake Salisbury (deep), Lake Manchester, Falling Creek (deep), and Swift Creek (deep) (see TABLE 7); and from Lake Anna III, Pseudomonas fluorescens was the least commonly encountered of five species isolated.

IMMUNOLOGICAL STUDIES

Although hyperimmune rabbit antisera were made against a variety of species of free-living amebas, notably N. fowleri (W. M. and T. Y. strains), N. gruberi, and Acanthamoeba castellanii, for reasons of time the sera were not utilized in characterizing such free-living amebas isolated. [Such studies are planned in the future; i.e., to test using indirect immunofluorescence (absorbed) the environmental strains isolated in Chang's liquid

media.]. Shortly after the study was commenced, it was realized by ourselves and others (10) that not all strains fluorescing with specific anti-Naegleria fowleri sera were pathogenic or were N. fowleri. Therefore, immunological tests were not considered critical to identifying pathogens; i.e., pathogenicity was defined only by demonstrating that an organism was indeed pathogenic (namely, it produced disease in an animal or at least CPE in a tissue culture cell line).

SPECIAL STUDIES

Studies Conducted by Virginia Polytechnic Institute and State University (VPISU) (See TABLES B-22 through B-42)

The results of all chemical tests are shown in TABLES B-22 through B-36, APPENDIX B). In general, the March, 1977, samples indicated that Falling Creek Reservoir and Lake Manchester were among the most enriched of the lakes examined. (Moore's Lake and Swift Creek Reservoir were not tested at this time.) These two lakes had some of the highest water levels of organic carbon (TABLE B-26), nitrogen, phosphorous, calcium, iron and magnesium recorded (TABLES B-23 through B-25). Sediments examined at this time from these two lakes also had high concentrations of carbon and nitrogen, as well as iron, magnesium and potassium (TABLES B-27 and B-28).

Lakes Overhill and Salisbury were below Falling Creek Reservoir and Lake Manchester in enrichment, while Lakes Pocahontas and Pallisades were the least enriched. Lake Overhill was different from all other lakes because of its high conductivity, which was reflected by the high hardness (80 mg/l), sulfates (76 mg/l), chlorides (147.5 mg/l), calcium (20 mg/l) and sodium (206 mg/l). The explanation(s) for this was probably the daily addition of chlorine, aluminum sulfate, and potassium carbonate by the owner.

The water chemistry data for Lakes Anna I, II, and III were similar at all sites, except for the chlorides and organic carbon which were higher at Lake Anna II. The water at all three sites were high in manganese and iron. Examinations of the sediment from all the Lakes Anna revealed that Lake Anna II and III were higher in ash-free weight, iron, and magnesium than was Lake Anna I. The high values in metals were believed due to scraping of the lake bottoms before inundation. The reason for the high chloride and organic carbon in the water for Lake Anna II was not apparent.

The results of chemical analyses of water and sediment obtained August 23, about six months after the first comprehensive studies, are shown in TABLES B-29 through B-36. (Moore's Lake and Swift Creek Reservoir were sampled at this time; however Pallisades Lake was not.) The results of an enrichment ranking of the lakes were similar to those in March, 1977; i.e., Falling Creek Reservoir, Lake Manchester, and Lake Salisbury appeared to be the most enriched, while Lake Pocahontas and Swift Creek Reservoir were almost as high. Moore's Lake and Lake Overhill appeared very different from other lakes sampled in that conductivity, chlorides, sulfates, and pH were very high, while organic carbon was low. Again, this was probably due to the addition of various chemicals (aluminum sulfate, chlorine, and potassium carbonate), especially during the month of August when people were

swimming. The sediment data (TABLES B-34 through B-36) also reflected the enrichment pattern established in the water.

Again all three sampling sites on Lake Anna appeared to have water that was very similar chemically. The similarity in water enrichment was reflected in the sediments, except at Lake Anna III where the ash-free dry weight, Fe, Mg, and K were higher than at Lakes Anna I or II.

In comparing the August data with the March data, the $\text{NH}_3\text{-N}$ and Org-N in the water were higher in August than in March for all lakes; however, the $\text{NO}_3\text{-N}$ was less. These observations suggested that the plankton in the lakes were using up the NO_3 at a higher rate in August than in March and converting NO_3 into organic material because of greater growth rates during the warmer months. In addition, in August the Fe and Mg concentrations in the water were higher than in March, probably due to low oxygen levels in the sediments which aided in converting these two ions into more soluble forms. In general, the data suggested that the lakes, with the exception of Moore's and Overhill, were more active biologically in August than in March, but otherwise were reasonably similar in their chemical characteristics from month to month.

Studies of the microbiota, especially of the protozoan communities, were conducted on Lakes Overhill, Salisbury, Pocahontas, Manchester, Anna, and Falling Creek, principally by studying plankton samples and polyurethane foam (PF) units that had been submerged for 14 days in each of the lakes (14,15). The number and variety of protozoans identified in plankton samples indicated that a significant imbalance among protozoans was present, as suggested by the absence of euglenoids in Lakes Overhill, Salisbury, and Fallisades (TABLE B-37). Large numbers of euglenoids usually have been associated with waters that contained relatively high concentrations of organic pollutants (24). Euglenoids were not dominant in any of the plankton samples examined, even though they were present in substantial numbers in the Lake Anna sites.

The number and variety of nonprotozoans found in plankton samples indicated that two noticeable imbalances were present (TABLE B-38): the low total numbers of nonprotozoans in Lake Overhill and the high number of desmids in Lake Salisbury. Some investigators have proposed that desmids are indicators of oligotrophy, provided consideration is given to both numbers and kinds (25). The low number and variety of species in Lake Overhill was probably due to the frequent addition of chemicals to this lake (vide supra).

The numbers of protozoan species that colonized PF units ranged from 44 in Lake Anna I to 21 in Lake Manchester (TABLE B-39). The distribution among groups revealed an imbalance in Lake Pocahontas caused by euglenoids and holotrichs; however, the number of individuals was not unusual. Many protozoans apparently have cosmopolitan distribution and broad physicochemical tolerances (26); consequently, the numbers of individuals and associated biota have to be considered if they are to be used as indicators. The dominant species, in terms of numbers of individuals per species, were limax amebas, which were impossible to identify without culturing each of them.

Lake Anna I was unusual; for it contained five sarcodines, each of which consisted of 100 or more individuals per slide (Amoeba sp., Mayorella sp., Amoeba gorgonia, Amoeba spumosa, and Cochliopodium bilimbosum). The Cochliopodium has been characteristic of many polysaprobic waters and anaerobiasis.

The total number of species in Lake Overhill was 23, or low, but numbers of individuals per species was high (Ca. - 100/slide) in the species Bodo caudatus, Bodo sp., Peranema trichophorum, Chilodonella uncinata and Vorticella microstoma, all of which have been characteristic of effluents from trickling filters and mixed liquor from activated sludge plants (27). Pseudospora sp., Acanthocystis aculeata and Cochliopodium bilimbosum also have been of this assemblage; in addition, the sulfur bacterium Achromatium has been present in large numbers.

Both the biflagellate and ameboid stages of Pseudospora were present. Since Pseudospora has been identified as a decimator of certain algae, this may have partially accounted for the low productivity seen in Lake Overhill.

Although the total number of nonprotozoan microbiota identified were higher in the PF units than in the plankton samples, the pattern was much the same in both (TABLE B-40). The total numbers ranged from 29 in Lake Pocahontas to 13 in Lake Overhill.

Lake Anna II ranked first according to the total number of planktonic protozoa (TABLE B-41), while Lake Anna I ranked first when studied with PF units (TABLE B-42). According to rankings based on organic carbon (TABLES B-26 and B-41), results were similar; i.e., Lake Anna II ranked first. However, even though Lakes Pocahontas, Falling Creek, and Anna I bore the same relationship to one another in both kinds of samples, the rankings did not correlate with organic carbon or the most enriched bodies of water according to physicochemical determinations (TABLES B-22 through B-36).

In Vitro Study of Effect of Lake Bacteria Against Pathogenic Amebas

A variety of bacterial species and strains obtained from environmental water sampling were tested in vitro against a known human pathogenic isolate (N. fowleri, W. M. strain) for inhibitory activity or for their inability to support the growth of the ameba as judged by plaque diameters (see TABLE 8). Enterobacter aerogenes, utilized by Wellings (10) in her laboratory as bacterial food for Naegleria was employed as a control. At an incubation temperature of 37°C, at 24, 48, and 72 hours results were similar for the control Enterobacter aerogenes, Enterobacter cloacae, Escherichia coli, and Pseudomonas aeruginosa. Klebsiella pneumoniae did appear to be an excellent source of food, as plaque diameters at all incubation times were greater than with any other bacteria utilized. At 72 hours the plaque diameter was almost one and one half larger than the control.

Perhaps more striking than the excellent amebic growth in the presence of Klebsiella pneumoniae, was the small plaque size seen with Pseudomonas fluorescens and the absence of any plaques (with the exception of 72 hours of incubation) seen when a pigmented Serratia marcescens was used as the

TABLE 8. MEAN PLAQUE DIAMETERS (MM) OF N. FOWLERI (W. M. STRAIN)
AGAINST A VARIETY OF ENVIRONMENTAL ISOLATES OF BACTERIA*

Incubation Time (hrs., 37°C	Enterobacter aerogenes (Wellings strain)	Enterobacter cloacae 25-1	Escher- ichia coli 102-1	Pseudo- monas aeruginosa 112-4	Pseudo- monas fluorescens 65-4	Serratia marcescens 105-3	Klebsiella pneumoniae 34-4
24	17 (15-22)†	14 (12-15)	16.8 (15-20)†	15.6 (15-17)	10.3 (0-13)	0 (0)‡	19.8 (17-21)†
48	32.8 (28-45)	31.3 (30-33)	32.4 (30-34)	29.8 (28-32)	16.6 (13-17)	0 (0)	43.6 (41-47)
72	46.4 (42-53)	45 (42-50)	47.4 (45-50)	43.0 (42-45)	30.2 (28-37)	4.2 (0-12)	69.0 (62-73)

* Numbers in parentheses represent range. Means were based on five experiments.

† 24 hour reading - large plaque may have been due to overfilling of well with amebas.

‡ Amebas were encysted and appeared to incorporate red pigment into cell walls. Amebas were not tested subsequently for viability.

TABLE 9. MEAN PLAQUE DIAMETERS (MM) OF N. FOWLERI (W. M. STRAIN)
AGAINST ENVIRONMENTAL ISOLATE OF SERRATIA MARCESCENS (STRAIN NO. 105-3)*

Incubation Time (hrs.)	<u>Enterobacter</u> <u>cloacae</u> control 25-1	<u>Serratia</u> <u>marcescens</u>	
		pigmented 105-3	non-pigmented mutant 105-3
24	14.8 (12-17)	0	6.2 (0-10)
48	27.6 (23-32)	0	14.8 (10-17)
72	42.0 (40-45)	0	21.6 (13-25)

* Numbers in parenthesis represent range. Means were based on five experiments.

sole nutrient. Although the experiments were repeated five times and the means compared, at no time did the greatest (maximum) diameter for Pseudomonas fluorescens or Serratia marcescens equal the smallest (minimum) plaque diameters with the other bacteria. With the use of Serratia marcescens, amebas encysted and appeared to incorporate the red pigment into their cell walls.

The explanation for the inhibition of amebas by certain species of bacteria or the lack of their utilization by amebas as a food source, was not apparent. Viability, proliferation, feeding, and motility, all of which may have been inhibited as reflected by small or absent plaques, were not individually studied. Chang has reported that certain bacteria, most notably pigment producing strains of Pseudomonas and Serratia, served as poor food sources for Naegleria gruberi (23).

The mean plaque diameters using the pigment producing environmental isolate of Serratia marcescens strain 105-3 and a nonpigmented mutant from it against the known human pathogen N. fowleri (strain W. M.) was also measured (TABLE 9). The parent pigmented strain completely inhibited the amebas, resulting in no plaques; while the nonpigmented mutant of 105-3 did support growth, albeit with plaque diameters half that of the control bacteria Enterobacter cloacae. Thus, it appeared that the red pigment prodigiosin produced by Serratia marcescens was toxic to pathogenic amebas or did not support their growth at all. Nevertheless, all inhibition did not appear to reside with the pigment, as Serratia marcescens without pigment also inhibited pathogenic amebas. However, the nonpigmented mutant was not tested chemically to determine whether or not portions of the pigment were contained. In addition, such things as optimum light, media, and incubation temperatures for pigment expression were not determined for the bacteria; so that pigment may have been present (perhaps in minute amounts) but was not detected.

Further studies were performed to confirm these findings (TABLES 10 and 11). Three strains of environmental isolates (numbers 105-3, 153-2, and 9-4) of pigmented Serratia were tested against the T. Y. strain of pathogenic N. fowleri and similar results were observed (TABLE 10). However, at 72 hours of incubation 153-2 and 9-4 were at times associated with sizeable plaque diameters. Again however, nonpigmented strains of Serratia isolated from fresh-water lakes also demonstrated inhibition or lack of growth support for amebas (TABLE 11). That indeed some of the nonpigmented strains could have been producing portions of the pigment molecule prodigiosin was suggested by the observation that mutant strain 9-3-3 (obtained from Baylor University) which produced only the bipyrrole pigment was completely inhibitory; whereas another mutant strain (WF) from the same parent, which produced only the monopyrrole pigment, supported growth well and was noninhibitory.

The significance of these findings was not completely clear, but the presence or absence of bacteria in the lake waters may well have influenced the number and variety of free-living amebas present in all the lakes studied. The number of bacteria necessary to inhibit amebic growth or destroy them was not determined, but it might be larger than exists. The number of

TABLE 10. MEAN PLAQUE DIAMETERS (MM) OF *N. FOWLERI* (T. Y. STRAIN)
USING PIGMENTED *SERRATIA* SP. ISOLATED FROM FRESH WATER LAKES AS THE SOLE FOOD SOURCE
(INOCULUM T. Y. = 1.73×10^4 AND BACTERIA 1.05×10^9 ; AGAR DEPTH 5-7 MM; INCUBATION 44°C)*

Isolate of Pigmented <i>Serratia</i>	No. of Plates	Hours of Incubation		
		24	48	72
105-3	1	0†	0†	0†
	2	0†	0†	0†
	3	0†	0†	0†
	4	0†	0†	0†
	5	0†	0†	0†
	6	8	12	25
153-2	1	0§	0§	15
	2	0†	0†	0†
	3	0§	0†	20
	4	0†	0†	30
	5	0†	0†	30
	6	0†	0†	30
9-4	1	0†	0†	0†
	2	0†	0†	20
	3	0†	0†	25
	4	0†	0†	0†
	5	0†	0†	20
	6	0†	0†	20

* For control *Enterobacter cloacae* was associated with mean plaque diameter of 14.8, 27.6, and 42 mm at 24, 48, and 72 hours, respectively.

† Amebas appeared to have ingested red pigment and fragmented.

‡ Healthy amebas in well and closely surrounding well.

§ Some fragmented amebas and some healthy amebas.

TABLE 11. MEAN PLAQUE DIAMETERS (MM) OF *N. FOWLERI* (T. Y. STRAIN)
USING NONPIGMENTED *SERRATIA* SP. ISOLATED FROM FRESH-WATER LAKES AS THE SOLE FOOD SOURCE
FOR AMEBAS (INOCULUM T. Y. = 1.73 x 10⁴; BACTERIA 1.05 x 10⁹; AGAR DEPTH 5-7 MM; INCUBATION 44°C)

Incubation Time (hrs.)	Control Enterobacter cloacae*	Non-pigmented <i>Serratia</i> Species Isolated From Fresh Water Lakes*					WF†	9-3-3†
		2-5	23-8	36-12	154-2	166-6		
24	29 (20-40)	2 (0-10)	10 (0-14)	0 (0)	0 (0)	8 (0-15)	26 (20-40)	0 (0)
48	37 (30-45)	3 (0-15)	14 (0-22)	0 (0)	4 (0-15)	18 (15-20)	49 (35-60)	0 (0)
72	48 (40-55)	8 (0-17)	23 (15-30)	0 (0)	6 (0-20)	23 (20-30)	61 (40-70)	0 (0)

* Numbers in parentheses represent range. Means based on six experiments.

† Represents strain of *Serratia marcescens* which produced only the monopyrrole pigment precursor.

‡ Represents strain of *Serratia marcescens* which produced only the bipyrrole pigment precursor.

TABLE 12. SUSCEPTIBILITY TESTS OF ENVIRONMENTAL PATHOGENIC ISOLATE NO. 931 (L. ANNA I, OBTAINED 10/4/78) AND KNOWN HUMAN PATHOGENIC STRAINS OF N. FOWLERI TO A VARIETY OF CHEMICAL AND NOXIOUS AGENTS*

Agent	N. fowleri; W. M.		N. fowleri; T. Y.		Strain No. 931	
	MIC	MAC (μ g/ml)	MIC	MAC (μ g/ml)	MIC	MAC (μ g/ml)
Amphotericin B	0.195	0.39	0.39	0.195	0.39	0.195
Amphotericin B	6.25	6.25	6.25	6.25	25.0	12.5
Methyl Ester	>50	>50	>50	>50	>50	>50
Minocycline	12.5	12.5	12.5	12.5	12.5	25.0
Furamide	0.3%	1.25%	not tested	not tested	0.3%	0.6%
NaCl	10%	10%	10%	10%	10%	10%
DMSO	25.0	25.0	25.0	25.0	>25.0	>25.0
Amphotericin B and Minocycline	0.39	0.78	0.39	1.56	0.78	1.56
SCH 16656	12.5	>12.5	12.5	>12.5	12.5	>12.5
Ketoconazole						

* MAC = Minimum Amebocidal Level or that concentration of the agent which effectively inhibited multiplication as recognized by absence of growth upon subculture in Nelson's medium in the absence of the agent. MIC = Minimum Inhibitory Concentration or that concentration which resulted in no visible organisms by 48 hours.

+ Amphotericin B and DMSO remained constant at .0975 μ g/ml and .078%, respectively.

bacteria used in the in vitro experiments conducted herein were in excess of the number or concentrations encountered naturally. Nevertheless, it was interesting that with the exception of Lake Anna III, no lake site from which Pseudomonas fluorescens was isolated has yielded pathogenic Naegleria at any time; and in the instance of Lake Anna III, the bacterium was the least common species isolated.

Study of Effect of Various Antiamoebic Agents Against Wild Strains of Pathogenic Amebas Isolated from Lakes

Studies of the susceptibility of a pathogenic environmental isolate (strain no. 931) against a variety of chemical and noxious agents revealed that the environmental isolate responded virtually identically to that of known human pathogenic strains W. M. and T. Y. of N. fowleri (TABLE 12). Membrane damaging agents such as the polyenes (amphotericin B and its methyl ester and SCH 16656) proved particularly effective, as did NaCl and DMSO. Thus, it appears as though the environmental strains of Naegleria are identical in susceptibility to chemotherapeutic agents as known human pathogens; however, more strains would have to be tested to confirm this finding.

Study of Effect of Prolonged Incubation of Samples at 44°C on Isolation of Amebas

Additional samples of bottom soil approximately 500 gms in size (similar to routine sampling) from Lake Anna III (sample no. 959) were subjected to prolonged incubation up to 1,000 hours at 44°C (see TABLE 13). Amebas were grown from 13 separate samples examined at varying time periods up to 1,000 hours incubation; however, amebas which would flagellate could only be isolated from seven specimens, none of which incubated longer than 600 hours. They were not isolated at 24 hours, but were at 288 hours. Pro-

TABLE 13. EFFECTS OF PROLONGED INCUBATION AT 44°C OF
BOTTOM SOIL SAMPLE NO. 959 FROM LAKE ANNA
III FOR THE ISOLATION OF PATHOGENIC AMEBAS
(COLLECTED ON 10/18/78; WATER TEMPERATURE 22.9°C)

Incubation time (hours)	Presence of amebas	Flagellation stage present	Growth in CSYECM Medium	Pathogenic for mice
24	+	-	-	-
288	+	+	+	++
312	+	+	+	++
336	+	+	+	++
360	+	+	+	-
384	+	-	-	-
400	+	+	++	-
500	+	+	-	-

(continued)

TABLE 13 (continued)

Incubation time (hours)	Presence of amebas	Flagellation stage present	Growth in CSYECM Medium	Pathogenic for mice
600	+	+	-	-
700	+	-	-	-
800	+	-	-	-
900	+	-	-	-
1000	+	-	-	-

* Growth in Chang's CSYECM Medium not sustained.

† 5/5 mice dead; N. fowleri reisolated from brain tissue.

longed incubation at 44°C appeared to enhance isolation of amebas which would grow in Chang's liquid (CSYECM) media, as well as pathogenic Naegleria. Nevertheless, pathogenic amebas were not isolated at 24 hour incubation, nor were they after 336 hours of incubation.

These studies were extended further in that 20 bottom soil samples were processed for varying time periods up to 500 hours (TABLE 14). Again, it was noteworthy that prolonged incubation at 44°C enhanced isolation of pathogenic forms, having not been identified in the bottom soil samples until 400 hours of incubation. The effect upon ameboflagellates did not appear significant.

The effect of prolonged incubation at 44°C was also studied by Wellings (10), who also noted that isolation of pathogenic forms of Naegleria were not adversely affected and selection perhaps enhanced. The reasons for this

TABLE 14. FREQUENCY OF ISOLATION OF AMEBAS AND AMEBOFLAGELLATES FROM 20 BOTTOM SOIL SAMPLES PROCESSED AFTER PROLONGED INCUBATION AT 44°C

	Hours of incubation at 44°C					
	24	100	200	300	400	500
Amebas	95%	90%	90%	90%	90%	80%
Ameboflagellates	10%	15%	10%	15%	25%	25%
Ameboflagellates isolated which grew in Chang's CSYECM	10%	5%	10%	0	10%	5%

TABLE 14 (continued)

	Hours of incubation at 44°C					
	24	100	200	300	400	500
Non-ameboflagellates isolated which grew in Chang's CSYECM	0	0	5%*	0	0	0
Pathogens isolated	0	0	0	0	5%	5%

* Growth not sustained.

are obscure, but perhaps cysts of pathogenic amebas were more resistant to drying and prolonged elevation of temperature than those of nonpathogenic Naegleria. Such findings might explain the relationship of elevated water temperatures (particularly persistently elevated water temperatures) to increased isolation of pathogenic Naegleria (vide supra, PHYSICOCHEMICAL STUDIES).

Study of Value of Sampling with a Plankton Net in an Attempt to Isolate Pathogenic Amebas

This study was undertaken to see if an easier yet reliable method of isolating pathogenic Naegleria could be identified which might be useful to individuals practicing in the field (see TABLE 15). Samples from a plankton net towed from a rowboat, however, were only 63% effective in isolating amebas and only one percent effective in yielding an ameba which would grow in Chang's media. (No pathogens were detected in any samples regardless of the method used.) Plankton net samples were not as effective as sampling water alone from a shallow area where 94% (15 of 16 samples) were positive for amebas which would grow at 44°C, and certainly not as effective as studying bottom soil samples, from which 100% (16 of 16) were positive for amebas, 50% were positive for amebflagellates, and 19% were positive for amebas which would grow in Chang's media. Thus, the technique of using or dragging a plankton net, although simple, was not believed reliable for testing for the presence of pathogenic forms, even though no pathogenic Naegleria were isolated at any time during this phase of the study.

TABLE 15. COMPARISON OF USING A PLANKTON NET RECOVERY TECHNIQUE WITH SHALLOW WATER
AND BOTTOM SOIL SAMPLING TECHNIQUES ON THE RECOVERY OF AMEBAS (A)
AND AMEBOFLAGELLATES (AF)

Lake	Sampling Date	Plankton Net			Shallow Water Column Sample			Shallow Mud Sample		
		A in Sample	AF	Growth in Chang's Medium	A in Sample	AF	Growth in Chang's Medium	A in Sample	AF	Growth in Chang's Medium
Salisbury	9/25	-	-	-	+	-	-	+	-	-
Swift Creek	9/28	+	-	-	+	-	-	+	+	+
	10/24	-	-	-	+	-	-	+	-	-
	11/7	+	-	-	+	-	-	+	+	-
	12/5	+	-	-	-	-	-	+	-	-
Pocahontas	10/2	+	-	-	+	-	-	+	+	-
	10/16	-	-	-	+	-	-	+	-	+
	10/30	-	-	-	+	-	-	+	+	-
	11/13	+	-	-	+	-	-	+	+	-
	11/28	+	-	-	+	-	-	+	-	-
Falling Creek	10/3	+	-	-	+	-	-	+	+	+
	10/17	-	-	-	+	-	-	+	-	-
	10/31	+	-	-	+	-	-	+	+	-
	11/14	-	-	-	+	-	-	+	+	-
	11/29	+	-	-	+	-	-	+	-	-
Anna	10/18	+	+	+	+	-	-	+	-	-
Totals		10 + (63%)	1 + (6%)	1 + (6%)	15 + (94%)	0	0	16 + (100%)	8 + (50%)	3 + (19%)

* Nonpathogenic for mice following intranasal instillation. Growth in Chang's Medium not sustained.

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APPENDIX A-1

CALF SERUM-CASEIN-YEAST EXTRACT MEDIUM FOR ANENIC CULTIVATION OF PATHO- GENIC NAEGLERIA

#1	500	1000
Isoelectric Casein	5.0	10.0 gms
Na ₂ HPO ₄ . 7 H ₂ O	1.25	2.5 gms
(if Na ₂ HPO ₄ anhydrous)	0.6625	1.325
Dissolve in Distilled water using a 2000 ml flask	400 ml	800 ml
#2		
Dextrose	1.25	2.5 gms
KH ₂ PO ₄	0.4	0.8
Dissolve in	40	80 ml
Autoclave #1 and #2 for 15 minutes and allow to cool		
#3		
Yeast extract powder	2.5	5.0 gms
Dissolve in of distilled water	10	20 ml
#4		
Fetal Calf Serum	50 ml	100 ml
#5		
Pencillin (stock)	0.5	1.0 ml
Streptomycin (stock)	0.5	1.0 ml

APPENDIX A-1 (CONTINUED)

Combine #'s 1, 2, 3, 4 and 5.

Disperse in 5-10 ml amounts into 15 X 125 screw capped tubes, aseptically.

Incubate for 2 days at 35° - 37° to test for sterility.

Make up #1 and #2 and autoclave.

Make up #3 and filter.

Pour #3 into #2. Pour #5 into #2. Pour #2 into #1.

Lastly, add #4.

APPENDIX A-2

PAGE'S SALINE *

NaCl	0.120 grams
MgSO ₄ .7H ₂ O	0.004 grams
CaCl ₂ .2H ₂ O	0.004 grams
Na ₂ HPO ₄	0.142 grams
KH ₂ PO ₄	0.136 grams

Deionized HOH - 1liter

* Page, F. C. 1967. Taxonomic criteria for limax amoebae with descriptions of 3 new species of Hartmanella and 3 of Vahlkampfia. J. Protozool. 14: 599-521.

APPENDIX A-3

NELSON'S MEDIUM*

Page's Saline (see APPENDIX A-2)

0.1% (W/V) Panmede Liver Digest

0.1% (W/V) Glucose

(Autoclave 15 lbs. psi, 15 min.)

2% (W/V) Calf Serum

Adjust pH 6.5

* Weik, R. R. and Johns, D. 1977. Agitated mass cultivation of Naegleria fowleri. J. Parasitol. 63:868-871, 1977.

APPENDIX A-4

PROTOCOL FOR OBTAINING WATER AND
BOTTOM SOIL SAMPLES AT LAKE SITES

1. A biweekly sampling schedule for each lake is utilized.
2. Prior to sampling, the hose for water removal is rinsed with fresh water from the lake for about one to two minutes (about eight liters).
3. Seventy-two (72) liters of water is removed from the sampling site via field water sampling assembly and filtered thru at 60 cm by 3.3 diameter column of Ottawa sand at a rate of 3 L/min. The sand is kept in the column contained by teflon tubing and returned to the laboratory.
4. A 300 ml sample of water from the same site is recovered and placed in a B.O.D. bottle and Winkler Iodometric Titration for dissolved oxygen is performed.
5. A 250 ml sample bottle is filled with water from the same site and placed in an ice cooler and returned to laboratory where pH is determined.
6. A 500 ml bottom soil sample is recovered via a large flat metal spoon, being certain only to remove a layer no deeper than 1 cm. This sample is placed in a zip-lock plastic bag and stored in our ice cooler until return to laboratory.
7. The temperature, electrical conductivity, and salinity of the water at the sampling site are measured and recorded.
8. For deep water samples, the same amounts of water are filtered from off the bottom (approximately three to four inches) and mud samples are removed by a special swing bucket apparatus. Samples are processed as above. Temperature, pH, dissolved oxygen, and conductivity are determined on bottom water samples from each site.
9. MPN's for total Gram negative bacilli are obtained by taking samples of lake water and putting them into bottles (150 ml) coated with sodium thiosulfate, placing the bottles in an ice cooler, and returning to the laboratory where studied EPA procedures for determining MPN's are performed.

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APPENDIX A-5

PROTOCOL FOR PROCESSING FIELD SAMPLES OF SOIL
FOR PATHOGENIC NAEGLERIA: BOTTOM SAMPLES

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1. About 500 grams of bottom sand from the sample sites are brought into the lab in plastic zip-lock bags. Each sample is numbered and logged into a book and incubated at 44°C overnight.
2. The 250 ml of one % beef extract (Difco #0126-01) and the sample are poured into a jar which is recapped and shaken vigorously.
3. The sample is then allowed to settle for at least 15 minutes. Then it is decanted into two 250 ml centrifuge bottles, and centrifuged for 30 minutes at 2500 RPM (900 g's) at 4°C in Beckman J-21 Fixed rotor. All but about 40 cc per bottle is decanted off.
4. Resuspend material by shaking by hand. (Note: the bottom sand, unlike Ottawa sand, does not settle out well in step 3; hence, a couple of inches of sand may be left in each centrifuge bottle. Make sure that at least the top part of the sediment is resuspended, on which cysts might be lying).
5. Pour into Falcon #2070 conical plastic centrifuge tubes and spin at 1500 RPM (650 g's) for 20 minutes at 4°C. Pour off supernatant except for about 2 ml and save pellet for inoculation.
6. Vortex vigorously for 5 seconds. Inoculate the sediment onto non-nutrient agar (2.5%) plates which have been seeded with live Enterobacter and allowed to dry at room temperature with the lids ajar. Incubate at 44°C in a metal canister with moisture plate and examine daily up to 5 days.
7. Examine after 24 hrs. for colonies of amebas. If amebas are present remove plug of agar containing amebas and place it into Chang's fluid axenic media (CSYECM) containing polymyxin B 100 µg/ml and/or gentamicin 100 µg/ml, and carbenicillin 200 µg/ml and incubate at 37°C for 24-72 hrs.
8. If luxuriant growth of amebas occur in Chang's liquid axenic media, put into mice for pathogenicity studies (see Mouse Inoculation Protocol for Pathogenicity Studies, Appendix A-8).
9. If no growth occurs in liquid media, plate is retained for future re-examination.*
10. If no growth occurs on plate, after 5 days, discard plate.

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* In some instances in which the presence of pathogenic amebas is strongly suspected (e.g., plaque line is sharp), amebas are transferred from primary plate to plate containing Enterobacter aerogenes or Enterobacter cloacae

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FOR TABLES
AND ILLUSTRATIONS

APPENDIX A-5 (continued)

and the resulting 48 hr. monaxenic culture inoculated intranasally into mice.

APPENDIX A-6

PROCESSING FIELD SAMPLES OF WATER FOR PATHOGENIC NAEGLERIA: SAND COLUMNS

1. In the field, 72 liters of lake water is filtered through a 61 cm column (I.D. 3.33 cm) of Ottawa #20-30 sand. Filtration rate 3.8 l/min. Volumes of water are the same for each lake sampled.
2. In the lab, number sample and log into book. Incubate columns overnight at 44°C. In the morning (18 hrs. later) pour sand into sterile one liter plastic container. Add 250 ml of one percent beef extract broth (Difco #0126-01). Shake vigorously for 3 minutes.
3. Allow sand to settle for at least 15 minutes. Decant off fluid into centrifuge bottles (250 ml). Concentrate fluid by centrifuging at 2500 RPM (950 x g) 30 min. and retain 80 ml supernatant and pellet (40 ml each bottle). Resuspend gently, place in Falcon #2070 conical centrifuge tubes and centrifuge at 2000 RPM for 30 min. (650 g's) at 4°C. Pour off or aspirate supernatant except for about 2 ml and save pellet for inoculation onto plates (see step 5).
4. Vortex pellet vigorously for five seconds and drop or pour the sediment (use applicator stick if necessary) onto nonnutrient agar (two percent) plates which have been seeded with a thick suspension of live Enterobacter cloacae and allow to dry at room temperature with lids ajar. Incubate at 44°C and hold for five days at 44°C before discarding.
5. Examine plates after 24 hours incubation and examine daily for colonies of amebas. If amebas are present, remove plug of agar containing amebas and place it into Chang's liquid axenic media containing polymyxin B 100 µg/ml, and/or gentamicin 100 µg/ml, and carbenicillin 200 µg/ml and incubate at 37°C for 24 to 72 hrs. Also remove plug from adjacent area and test for flagellates (see Flagellates Protocol). Save primary plate for future use.
6. If luxuriant growth of amebas occurs in Chang's liquid axenic media, put into mice for pathogenicity studies (see Mouse Inoculation Protocol for Pathogenicity Studies, Appendix A-8).
7. If no growth occurs in liquid media, plate is retained for possible future use or reexamination.*
8. If no growth occurs on plate after five days, discard plate.

* In some instances in which the presence of pathogenic amebas are strongly suspected (e.g., plaque line is sharp), amebas are transferred from primary plate to plate containing Enterobacter cloacae, and the resulting 48 hour monaxenic culture inoculated intranasally into mice.

APPENDIX A-7

FLAGELLATION PROTOCOL

1. From the original isolation plate (large monaxenic seeded with live Enterobacter and incubated at 44°C for 1-5 days), remove a piece of agar from area containing amoebae and transfer to agar plates seeded with live Enterobacter. Incubate at 44°C overnight.
2. After 18-24 hours, pick a small plug of agar containing amebas to 0.5 ml sterile distilled water in a small (Falcon #2054, 12 x 75 mm) plastic-capped tube (Wellings Method). Incubate at 37°C. Examine microscopically at one and one-half to two hours for flagellation. Keep examining throughout the day for flagellates.
3. Using the same plate from step 2, flood with sterile distilled water, and incubate at 37°C. Examine microscopically at one and one-half to two hours for flagellation and throughout the day.
4. Two monaxenic cultures of each ameboflagellate are retained - one at refrigerator temperature and one at room temperature. (Refer to subsection of log book entitled "subcultures".)

APPENDIX A-8

MOUSE INOCULATION PROTOCOL FOR PATHOGENICITY STUDIES

1. Instill a 0.01 ml suspension containing 10^4 amebas in Chang's liquid-axenic media (CSYECM) into the nasal cavity of ten Dublin (ICR) three-week old male mice which have been lightly anesthetized with intra-peritoneal ether.
2. Observe for four weeks postinoculation before discarding or interpreting as negative and interpreting amebas as nonpathogenic.

Note: Mice intranasally inoculated with pathogenic amebas generally will ruff, become unstable or somewhat paralyzed, circle, and die about five to seven days postinoculation. However, if inocula of pathogenic amebas are low, incubation periods may be longer.

3. The brain of dead mice are removed aseptically and cultured at 37°C for amebas by placing the whole brain in a Leighton tube containing 5 ml of Chang's liquid axenic media and placing tubes at 30° angles with lids loose. Tubes are examined daily for seven days before being recorded as negative.
4. The percent mortality due to amebas is recorded.
5. If amebas are isolated from brain, they are studied morphologically and stored for future study.

APPENDIX A-9

FLUORESCENT ANTIBODY (FA) PROTOCOL FOR
STUDY OF PATHOGENIC AMOEBAE

1. One drop of a culture suspension of the amoebae in question is placed on a glass slide, air dried at 43°C, fixed with methanol for three minutes, drained off, and allowed to air dry.
2. Slides are then placed horizontally in a moisture chamber.
3. Appropriately absorbed, diluted rabbit antisera is applied to the slide containing the organism in question for 30 minutes at room temperature (23°C) in a moisture chamber.
4. The sera is then drained off and the slide is placed in a vertical rack in a staining dish immersed in phosphate buffered saline (PBS) for ten minutes with agitation and with one change of PBS after five minutes.
5. Remove slides and return to moisture chamber and flood with commercially prepared fluorescein labeled goat antirabbit 7s globulin (Mely Lab, Springfield, Va.) 1:20 dilution which has been absorbed with nonpathogenic Naegleria and Acanthamoeba.
6. Allow slides to remain in the dark for 30 minutes at room temperature (23°C).
7. Drain off slides and return to vertical rack and immerse in staining dish containing PBS for 60 minutes with agitation included two changes of PBS. Cover to keep dark.
8. Remove slides and drain (do not blot). Air dry.
9. When dry, add one drop glycerin-PBS (9:1) and cover slip. Examine slides on FA scope.
10. A positive and negative control is always run simultaneously.

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APPENDIX B

TABLES

TABLE B-1. SUMMARY OF ISOLATES INOCULATED INTO MICE FOR PATHOGENICITY STUDIES

Date sample taken	Sampling site no.*	Type of sample	Lab no. of ameba isolated	Flagel- lated	Inoculum from CSYECM†	Inoculum size x 10 ⁴	No. mice died/ No. mice inoculated‡	Patho- genic
12/3/76	2	Bot. soil	1	+	No	1	0/10	-
12/16/79	7	Bot. soil	15	+	No	1	1/10s	-
1/3/77	6	Bot. soil	32	+	No	1	0/9	-
1/10/77	10	Bot. soil	37	+	No	1	0/10	-
1/11/77	2	Bot. soil	40	+	No	1	0/10	-
---	7	Bot. soil	---	+	No	1	0/10	-
---	2	Water	---	+	No	1	0/10	-
6/20/77	5	Water	223	+	No	3	4/10	+
6/27/77	11	Bot. soil	234	+	No	3	3/10	+
6/27/77	11	Water	236	+	No	4	1/10	+
7/25/77	5	Water	299	+	No	1	3/10	+
10/24/77	6	Bot. soil	445	-	No	5	0/10	-
1/9/77	3	Bot. soil	478	+	No	1.3	0/10	-
12/19/77	6	Bot. soil	537	+	Yes	23	0/10	-
1/11/78	8	Bot. soil	556	-	Yes	6.5	0/10	-
1/18/78	2	Bot. soil	561	+	Yes	4.1	0/10	-
1/18/78	3	Bot. soil	562	+	Yes	4.8	2/10s	-
4/19/78	17	Bot. soil	668	-	Yes	0.4	0/7	-
4/26/78	4	Water	679	-	Yes	0.2	0/10	-
5/17/78	14	Bot. soil	709	+	Yes	0.5	0/10	-
6/1/78	4	Bot. soil	742	+	No	3.8	1/5	+
6/1/78	8	Bot. soil	743	+	No	4.3	0/5	-
6/14/78	13	Bot. soil	772	+	Yes	2.5	0/10	-
7/11/78	2	Bot. soil	792	-	Yes	3	0/10	-

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TABLE B-1 (continued)

Date Sample taken	Sampling site no.*	Type of sample	Lab no. of ameba isolated	Flagel- lated	Inoculum from CSYECM†	Inoculum size x 10 ⁴	No. mice inoculated‡	Patho- genic
7/26/78	1	Bot. soil	817	+	Yes	3.7	0/10	-
7/31/78	6	Bot. soil	821	+	Yes	3.5	0/10	-
7/31/78	13	Bot. soil	822	+	Yes	4	0/10	-
8/1/78	11	Bot. soil	825	-	Yes	0.2	0/5	-
8/1/78	12	Bot. soil	826	-	Yes	0.3	0/5	-
8/17/78	3	Water	856	+	Yes	3	10/10	+
8/17/78	2	Bot. soil	858	+	Yes	1.1	10/10	+
8/22/78	8	Water	866	+	Yes	0.3	5/5	+
8/22/78	7	Bot. soil	868	-	Yes	1.4	0/5	-
8/30/78	2	Bot. soil	893	+	Yes	1.2	5/5	+
8/30/78	3	Bot. soil	894	+	Yes	3	10/10	+
8/30/78	4	Bot. soil	895	-	Yes	1.5	0/5	-
9/11/78	2	Bot. soil	909	+	Yes	4	8/10	+
9/28/78	11	Bot. soil	918	+	Yes	1.9	0/10	-
10/2/78	5	Bot. soil	922	+	Yes	1	0/5	-
10/2/78	18	Bot. soil	923	+	Yes	3.3	0/10	-
10/4/78	2	Bot. soil	931	+	Yes	3.5	5/5	+
10/9/78	14	Bot. soil	935	-	Yes	6	1/5§	-
10/16/78	5	Bot. soil	948	-	Yes	2	0/5	-
10/17/78	17	Bot. soil	953	-	Yes	2	0/5	-
10/18/78	2	Bot. soil	957	+	Yes	2.3	0/5	-
10/25/78	2	Bot. soil	969	+	Yes	5.7	1/10§	-
10/25/78	3	Bot. soil	970	+	Yes	6	0/10	-
11/6/78	14	Bot. soil	987	+	Yes	3.8	0/5	-
1/23/79	17	Bot. soil	1077	+	Yes	6.5	0/5	-
1/30/79	11	Bot. soil	1088	+	Yes	0.5	0/5	-
1/31/79	3	Bot. soil	1094	+	Yes	14	0/5	-
2/6/79	3	Bot. soil	1104	+	Yes	0.5	0/5	-
3/1/79	3	Bot. soil	1122	+	Yes	0.9	5/5	+
3/6/79	8	Bot. soil	1126	-	Yes	1	0/5	-

(continued)

TABLE B-1 (continued)

Date sample taken	Sampling site no.*	Type of sample	Lab no. of ameba isolated	Flagel- lated	Inoculum from CSYECM†	Inoculum size x 10 ⁴	No. mice inoculated‡	Patho- genic
4/27/79	3	Bot. soil	1144	+	Yes	3.9	0/5	-
6/19/79	2	Bot. soil	1238	+	Yes	1.4	0/5	-
6/19/79	3	Bot. soil	1239	+	Yes	1.0	0/5	-
8/11/79	2	Water	1291	+	Yes	1.0	0/5	-
8/11/79	2	Bot. soil	1294	+	Yes	1.1	0/5	-

* 1 = L. Overhill; 2 = Anna I; 3 = Anna II; 4 = Anna III; 5 = L. Pocahontas (shallow); 6 = L. Salisbury (shallow); 7 = Manchester; 8 = Falling Creek Reservoir (shallow); 9 = Moore's L.; 10 = Pallisades L.; 11 = Swift Creek Reservoir (shallow); 12 = Swift Creek Reservoir (deep); 13 = L. Salisbury (deep); 14 = L. Overhill (feeder); 17 = Falling Creek Reservoir (deep); 18 = L. Pocahontas (deep); 19 = Moore's L. (deep).

† CSYECM represents Chang's calf serum-yeast-extract-casein medium.

‡ Six week old were used from 12/3/76 through 1/18/78. Thereafter, weanling mice were utilized.

§ Mouse either died immediately after inoculation (probably from anesthesia), or amebas were not isolated from the brain post mortem.

** Not recorded.

TABLE B-2. SUMMARY OF MEAN MONTHLY WATER TEMPERATURES (°C)
FOR EACH LAKE SITE DURING 1977.

Site	Months											
	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
Overhill	-0.1	2.5				24.0	31.0	27.5	23.5	12.8	19.5	4.8
Overhill Feeder												5.0
Anna I	3.5	5.9	8.9	21.0	23.5	24.0	31.0	33.0	26.0	17.0	16.0	9.8
Anna II	3.0	6.0	10.0	19.5	24.5	23.0	32.0	30.8	25.0	14.3	17.0	6.8
Anna III	0.0	5.0	14.0	19.5	26.0	23.0	32.0	29.0	24.0	14.5	18.0	6.3
Pocahontas Shallow	0.3	11.4	18.5	23.5	22.5	31.5	30.3	28.5	28.0	17.0	8.8	2.0
Pocahontas Deep							25.0	27.0	22.8	15.8	8.5	
Salisbury Shallow	0.8	7.8	17.0	23.5	22.5	29.5	34.5	27.6	26.5	18.7	15.3	7.5
Salisbury Deep						29.5	31.0	28.0	26.0	16.3	15.0	7.3
Manchester	2.0	5.8	18.8	22.5	21.5	27.0	33.5	30.2	28.0	18.0	9.5	4.5
Falling Creek Shallow	1.9	5.5	17.7	21.5	20.0	29.0	31.0	29.2	26.3	21.5	12.7	4.0
Falling Creek Deep						12.4	12.0	13.7	16.3	16.5	12.0	3.5
Moore's Shallow							29.0	27.5	25.3			
Moore's Deep							30.0	27.8	28.3			
Pallisade's	1.8	5.3	12.5	18.0	28.0							
Swift Creek Shallow						31.0	36.5	31.9	29.0	19.8	15.5	6.8
Swift Creek Deep						26.7	30.5	28.4	26.0	17.8	14.6	6.5

TABLE B-3. SUMMARY OF MEAN MONTHLY WATER TEMPERATURES ($^{\circ}\text{C}$)
FOR EACH LAKE SITE DURING 1978.

Site	Months											
	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
Overhill	1.0	3.5	10.5			27.0	28.0	28.0		15.2	12.5	13.0
Overhill Feeder	0.7	3.0	9.5	14.0	21.2	30.5	28.0	27.6				
Anna I	3.2	3.5	4.8	12.2	17.0	24.5	32.4	34.8	34.1	23.6	22.1	18.3
Anna II	0.0	3.0	4.0	11.5	20.0	24.5	31.0	34.8	33.9	22.6	20.6	16.9
Anna III	0.0	2.3	1.5	10.5	21.0	25.5	28.0	30.9	28.3	16.5	17.3	14.9
Pocahontas Shallow	3.0	2.0		12.0	17.7	28.0	29.0	25.0	25.9	16.5	10.8	
Pocahontas Deep	2.0			11.5	18.0	24.5	28.0	24.5	22.1	14.5	9.5	
Salisbury Shallow	4.5	6.5	3.0	15.6	25.2	24.5	27.7	28.1				
Salisbury Deep		5.2	3.0	14.4	19.8	23.0	23.2	27.5				
Manchester	4.5	3.7	16.0	12.0	22.0	26.0	26.3	31.7	26.9			
Falling Creek Shallow	2.5	3.4	7.4	14.0	12.5	25.0	29.0	29.3	28.2	17.9	12.4	
Falling Creek Deep	0.0	3.8	6.0	13.0	10.5	19.5	14.7	14.0	15.0	14.9	10.9	
Moore's Shallow												
Moore's Deep												
Pallisade's												
Swift Creek Shallow	2.2	6.0	4.0	18.8	24.8	26.5	33.0	31.1		19.9	17.4	11.1
Swift Creek Deep	2.0		4.0	15.0	20.1	25.0	28.0	28.8		19.5	15.1	11.1

TABLE B-4. SUMMARY OF MEAN MONTHLY WATER TEMPERATURES (°C)
FOR EACH LAKE SITE DURING 1979.

Site	Jan.	Feb.	Mar.	Apr.	May	Months				Oct.	Nov.	Dec.	
						Jun.	Jul.	Aug.	Sep.				
Overhill													
Overhill Feeder	7.6	3.4		19.1	23.2	21.8	25.7	28.4					
Anna I	11.2	10.9	12.7	14.6	26.4	29.8	30.1	20.0					
Anna II	9.4	10.2	13.4	15.6	24.2	29.0	30.0	31.2					
Anna III	7.6	4.6	10.8	16.2	21.3	24.1	30.0	29.4					
Pocahontas Shallow				15.8	19.4	20.7	26.0	27.0					
Pocahontas Deep				14.9	18.4	20.1	21.3	26.8					
Salisbury Shallow				16.7	23.3		27.0	25.0					
Salisbury Deep				15.1	20.3		23.4	24.4					
Manchester							25.1						
Falling Creek Shallow	6.7	4.2	12.7	17.2	22.1	20.9	22.9	28.7					
Falling Creek Deep	5.2	3.5	6.2	12.6	12.6	12.8	11.5	15.9					
Moore's Shallow													
Moore's Deep													
Pallisade's													
Swift Creek Shallow	5.3	6.2		16.1	22.7		24.2	27.1					
Swift Creek Deep	5.4			17.4	21.1		28.0	25.3					

TABLE B-5. SUMMARY OF MEAN MONTHLY DISSOLVED OXYGEN (PPM)
FOR EACH LAKE SITE DURING 1977

Site	Months											
	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
Overhill	11.6	12.8	11.6			9.0	7.4	7.8	11.0	10.6	11.5	11.4
Overhill Feeder												11.5
Anna I	12.8	12.5	11.3	10.0	9.2	8.4	7.8	6.5	7.6	7.5	9.0	9.9
Anna II	13.0	12.6	11.6	10.2	9.5	9.0	7.5	6.9	7.9	7.8	8.8	10.9
Anna III	14.7	13.5	10.5	10.5	9.0	7.9	7.5	7.6	8.0	8.2	8.5	11.1
Pocahontas Shallow	14.0	11.7	10.1	9.3	9.1	8.4	8.0	8.5	9.0	8.4	10.3	13.0
Pocahontas Deep							1.4	2.2	3.0	6.9	10.0	
Salisbury Shallow	14.9	11.4	11.1	9.1	8.6	8.1	8.8	7.3	7.4	9.4	8.7	11.0
Salisbury Deep						7.7	7.2	6.7	7.5	8.3	12.5	10.5
Manchester	16.5	14.5	10.0	9.9	8.3	9.3	17.3	7.1	9.0	9.2	11.5	13.4
Falling Creek Shallow	14.5	13.2	11.0	10.8	8.5	7.7	8.3	7.7	8.9	7.4	8.4	13.0
Falling Creek Deep						0.4	0.0	0.0	0.0	0.0	3.8	0.0
Moore's Shallow							7.9	8.1	8.8			
Moore's Deep								7.7	8.8			
Pallisade's	13.8	14.5	10.5	9.5	9.6							
Swift Creek Shallow						9.3	9.2	9.4	10.2	10.3	9.5	11.0
Swift Creek Deep						7.6	6.9	7.3	7.5	9.0	8.5	10.5

TABLE B-6. SUMMARY OF MEAN MONTHLY DISSOLVED OXYGEN (PPM)
FOR EACH LAKE SITE DURING 1978

Site	Months											
	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.*	Oct.*	Nov.*	Dec.*
Overhill	12.0	11.2	10.8	ND†	ND†	7.5	7.2	7.6				
Overhill Feeder	12.2	11.8	10.8	9.8	7.6	6.5	4.3	5.3		5.3	6.1	6.4
Anna I	11.0	12.2	12.2	9.5	9.5	8.0	6.5	6.0	5.9	5.8	6.3	7.0
Anna II	11.5	11.9	12.5	9.5	9.0	7.5	6.3	6.5	6.9	5.8	6.7	7.1
Anna III	12.0	12.3	13.2	9.3	7.3	7.4	6.0	5.7	5.5	6.5	6.9	7.3
Pocahontas Shallow	12.0	11.8	ND†	9.8	9.2	6.8	6.7	6.3	6.3	6.6	6.8	
Pocahontas Deep				9.7	8.3	7.5	3.1	5.4	3.8	4.7	5.5	
Salisbury Shallow	10.5	10.5	12.4	9.5	8.3	8.0	7.6	6.9	7.7			
Salisbury Deep		11.6	12.3	8.8	8.0	8.2	6.7	6.0	6.3			
Manchester	10.8	11.4	10.5	9.8	7.9	7.8	7.2	6.8	7.7			
Falling Creek Shallow	11.7	11.6	11.2	9.3	9.0	7.3	7.8	8.1	9.1	5.8	5.8	
Falling Creek Deep	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.2	5.0	
Moore's Shallow												
Moore's Deep												
Pallisade's												
Swift Creek Shallow	11.5	11.2	11.5	9.3	7.2	7.5	7.5	8.6	7.9	7.3	7.9	7.3
Swift Creek Deep	12.5		11.0	9.4	8.1	7.2	6.3	6.0	5.1	6.0	6.4	7.3

* Error in method discovered. Results deleted from summary and not included in Table 4 or Figure 19.
† Not determined.

TABLE B-7. SUMMARY OF MEAN MONTHLY DISSOLVED OXYGEN (PPM)
FOR EACH LAKE SITE DURING 1979

Site	Months											
	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.*	Oct.*	Nov.*	Dec.†
Overhill												
Overhill Feeder					ND†	ND†	8.3	8.7				
Anna I	6.5			7.8	7.0	4.5	4.6	3.9				
Anna II	6.0			10.6	9.0	7.3	6.2	7.0				
Anna III	6.1			10.4	9.0	7.6	6.3	7.1				
Pocahontas Shallow	6.4			10.6	9.3	7.9	7.7	7.5				
Pocahontas Deep				8.8	9.0	7.8	8.2	7.3				
Salisbury Shallow				8.4	8.4	7.7	6.0	5.2				
Salisbury Deep				10.4	9.4	ND†	8.1	7.8				
Manchester				10.2	8.8	ND†	7.9	ND†				
Falling Creek Shallow	6.9			11.6	12.1	9.2	8.4	8.4				
Falling Creek Deep	5.8			6.7	1.1	0.0	0.0	0.0				
Moore's Shallow												
Moore's Deep												
Pallisade's												
Swift Creek Shallow	8.1			11.7	9.6	ND†	9.5	8.0				
Swift Creek Deep	7.5			9.3	9.4	ND†	7.7	5.7				

* Error in method discovered. Results deleted from summary and not included in Table 4 or Figure 19.
† Not determined.

TABLE B-8. BACTERIAL FLORA OF LAKE OVERHILL
(SITE NO. 1)

Sampling Date	MPN	Fecal MPN	Predominant Gram Neg. Bacilli*
11/9/77	430	93	N.D.
12/7/77	4	4	1. <u>Pseudomonas fluorescens</u> 2. <u>Serratia marcesens</u>
12/14/77	3	3	N.D.
1/11/78	23	4	N.D.
1/25/78	4	4	N.D.
3/14/78	3	3	1. <u>Pseudomonas species</u>
6/12/78	93	3	N.D.
7/26/78	3	3	N.D.

* N.D. = Not Determined; Number preceding species name indicates order of predominance; i.e., "1" most predominant; "2" next most predominant; etc...

TABLE B-9. BACTERIAL FLORA OF LAKE OVERHILL FEEDER
(SITE NO. 14)

Sampling Date	MPN	Fecal MPN	Predominant Gram Neg. Bacilli*
12/14/77	43	3	N.D.
1/11/78	640	9	1. <u>Escherichia coli</u> 2. <u>Enterobacter cloacae</u>
1/25/78	2400	2400	1. <u>Enterobacter cloacae</u>
3/14/78	120	3	1. <u>Enterobacter cloacae</u>
4/17/78	10	3	1. <u>Enterobacter cloacae</u>
5/2/78	750	140	N.D.
6/2/78	4600	93	N.D.
7/26/78	24000	43	N.D.

* N.D. = Not Determined; Number preceding species name indicates order of predominance; i.e., "1" most predominant; "2" next most predominant; etc...

TABLE B-10. BACTERIAL FLORA OF LAKE ANNA I
(SITE NO. 2)

Sampling Date	MPN	Fecal MPN	Predominant Gram Neg. Bacilli*
11/9/77	43	23	N.D.
12/21/77	230	9	1. <u>Klebsiella pneumoniae</u>
1/16/78	930	4	1. <u>Klebsiella pneumoniae</u> 2. <u>Citrobacter freundii</u>
2/22/78	43	21	N.D.
3/9/78	N.D.	1,100	N.D.
4/13/78	3	23	1. <u>Pseudomonas species</u>
6/1/78	75	3	N.D.
7/11/78	230	23	N.D.
8/2/78	230	3	N.D.

* N.D. = Not Determined; Number preceding species name indicates order of predominance; i.e., "1" most predominant; "2" next most predominant, etc...

TABLE B-11. BACTERIAL FLORA OF LAKE ANNA II
(SITE NO. 3)

Sampling Date	MPN	Fecal MPN	Predominant Gram Neg. Bacilli*
11/9/77	150	93	N.D.
12/7/77	43	3	N.D.
12/21/77	24,000	23	1. <u>Proteus morganii</u> 2. <u>Enterobacter cloacae</u> 3. <u>Escherichia coli</u>
1/16/78	9	3	N.D.
2/22/78	460	15	1. <u>Enterobacter cloacae</u>
3/9/78	1,100	N.D.	N.D.
4/13/78	3	9	1. <u>Serratia rubidae</u>
6/2/78	93	4	1. <u>Enterobacter cloacae</u>
7/11/78	750	3	N.D.
8/2/78	24,000	9	N.D.

* N.D. = Not Determined; Number preceding species name indicates order of predominance; i.e., "1" most predominant; "2" next most predominant, etc...

TABLE B-12. BACTERIAL FLORA OF LAKE ANNA III
(SITE NO. 4)

Sampling Date	MPN	Fecal MPN	Predominant Gram Neg. Bacilli*
11/9/77	230	23	N.D.
12/7/77	23	40	1. <u>Enterobacter cloacae</u> 2. <u>Klebsiella pneumoniae</u>
12/21/77	150	4	1. <u>Enterobacter cloacae</u> 2. <u>Serratia marcescens</u>
1/16/78	93	3	1. <u>Enterobacter cloacae</u>
2/22/78	93	4	N.D.
4/13/78	3	39	1. <u>Enterobacter hafniae</u> 2. <u>Pseudomonas fluorescens</u>
6/2/78	430	90	N.D.
7/11/78	93	3	N.D.
8/2/78	750	93	N.D.

* N.D. = Not Determined; Number preceding species name indicates order of predominance; i.e., "1" most predominant; "2" next most predominant, etc...

TABLE B-13. BACTERIAL FLORA OF LAKE POCAHONTAS
(SITE NO. 5)

Sampling Date	MPN	Fecal MPN	Predominant Gram Neg. Bacilli*
11/15/77	930	430	N.D.
11/30/77	1,500	210	1. <u>Escherichia coli</u>
12/13/77	930	430	1. <u>Serratia marcescens</u> 2. <u>Citrobacter freundii</u>
1/9/78	24,000	230	N.D.
1/23/78	2,400	430	1. <u>Enterobacter cloacae</u> 2. <u>Escherichia coli</u>
2/8/78	2,400	N.D.	N.D.
4/18/78	930	230	1. <u>Escherichia coli</u> 2. <u>Enterobacter cloacae</u>
5/3/78	2,400	93	N.D.
5/23/78	750	93	1. <u>Klebsiella pneumoniae</u>
6/5/78	390	230	N.D.
6/27/78	24,000	1,500	N.D.
7/18/78	21	230	N.D.
7/24/78	930	15	N.D.

* N.D. = Not Determined; Number preceding species name indicates order of predominance; i.e., "1" most predominant; "2" next most predominant, etc...

TABLE B-14. BACTERIAL FLORA OF LAKE POCAHONTAS (DEEP)
(SITE NO. 18)

Sampling Date	MPN	Fecal MPN	Predominant Gram Neg. Bacilli*
11/15/77	430	75	1. <u>Klebsiella pneumoniae</u>
1/9/78	430	93	1. <u>Enterobacter cloacae</u>
1/23/78	430	75	N.D.
4/18/78	930	43	N.D.
5/3/78	4,600	93	N.D.
5/23/78	130	43	N.D.
6/5/78	930	15	N.D.
6/27/78	11,000	1,500	N.D.
7/18/78	230	4,600	N.D.
7/24/78	2,400	43	N.D.

* N.D. = Not Determined; Number preceding species name indicates order of predominance; i.e., "1" most predominant; "2" next most predominant; etc...

TABLE B-15. BACTERIAL FLORA OF LAKE SALISBURY
(SITE NO. 6)

Sampling Date	MPN	Fecal MPN	Predominant Gram Neg. Bacilli*
11/7/77	11000	930	1. <u>Proteus morganii</u> 2. <u>Escherichia coli</u>
11/21/77	75	9	1. <u>Escherichia coli</u>
12/5/77	24000	43	1. <u>Enterobacter cloacae</u>
12/19/77	430	93	N.D.
1/30/78	210	23	N.D.
2/21/78	93	15	1. <u>Pseudomonas fluorescens</u>
3/8/78	29	3	N.D.
4/12/78	15	43	1. <u>Escherichia coli</u>
4/25/78	20	3	1. <u>Enterobacter cloacae</u>
5/11/78	230	11	1. <u>Escherichia coli</u> 2. <u>Pseudomonas fluorescens</u>
5/22/78	150	23	N.D.
6/14/78	11000	230	N.D.
7/17/78	930	430	N.D.
7/31/78	24000	150	N.D.
8/15/78	11000	1500	N.D.

* N.D. = Not Determined; Number preceding species name indicates order of predominance; i.e., "1" most predominant; "2" next most predominant; etc...

TABLE B-16. BACTERIAL FLORA OF LAKE SALISBURY (DEEP).
(SITE NO. 13)

Sampling Date	MPN	Fecal MPN	Predominant Gram Neg. Bacilli*
11/7/77	11000	430	1. <u>Pseudomonas aeruginosa</u> 2. <u>Proteus morganii</u>
11/21/77	43	4	1. <u>Enterobacter aerogenes</u> 2. <u>Serratia marcescens</u>
12/5/77	430	9	1. <u>Serratia marcescens</u> 2. <u>Enterobacter cloacae</u>
12/19/77	430	230	N.D.
2/21/78	23	3	1. <u>Pseudomonas fluorescens</u>
3/8/78	21	4	1. <u>Pseudomonas fluorescens</u>
4/12/78	9	43	1. <u>Escherichia coli</u>
4/25/78	43	23	1. <u>Serratia marcescens</u> 2. <u>Escherichia coli</u>
5/22/78	43	23	N.D.
6/14/78	93	15	N.D.
6/14/78	2400	430	N.D.
7/17/78	11000	2400	N.D.
7/31/78	24000	1500	N.D.
8/15/78	11000	2100	N.D.

* N.D. = Not Determined; Number preceding species name indicated order of predominance; i.e., "1" most predominant; "2" next most predominant; etc...

TABLE B-17. BACTERIAL FLORA OF LAKE MANCHESTER
(SITE NO. 7)

Sampling Date	MPN	Fecal MPN	Predominant Gram Neg. Bacilli*
11/16/77	430	430	N.D.
11/30/77	2400	430	1. <u>Escherichia coli</u> 2. <u>Klebsiella pneumoniae</u>
12/12/77	23	3	1. <u>Pseudomonas fluorescens</u> 2. <u>Enterobacter cloacae</u>
1/10/78	24000	75	1. <u>Klebsiella pneumoniae</u> 2. <u>Escherichia coli</u>
1/24/78	43	3	1. <u>Enterobacter cloacae</u>
2/8/78	43	N.D.	N.D.
2/27/78	240	93	1. <u>Escherichia coli</u>
3/15/78	23	23	N.D.
4/19/78	1100	1100	1. <u>Escherichia coli</u> 2. <u>Enterobacter cloacae</u>
5/2/78	460	240	N.D.
6/2/78	930	430	N.D.
6/13/78	430	230	N.D.
7/12/78	2100	2100	N.D.
7/25/78	24000	4600	N.D.

* N.D. = Not Determined; Number preceding species name indicates order of predominance; i.e., "1" most predominant; "2" next most predominant; etc...

TABLE B-18. BACTERIAL FLORA OF LAKE FALLING CREEK
(SITE NO. 8)

Sampling Date	MPN	Fecal MPN	Predominant Gram Neg. Bacilli*
11/2/77	430	23	1. <u>Escherichia coli</u>
11/14/77	930	93	N.D.
11/29/77	4600	430	1. <u>Escherichia coli</u> 2. <u>Enterobacter cloacae</u> 3. <u>Klebsiella pneumoniae</u>
11/12/77	1500	93	1. <u>Escherichia coli</u> 2. <u>Enterobacter cloacae</u>
1/10/78	24000	2400	1. <u>Serratia liquifacens</u> 2. <u>Serratia species</u> 3. <u>Klebsiella pneumoniae</u>
1/24/78	43	3	1. <u>Enterobacter cloacae</u>
2/6/78	230	15	N.D.
2/28/78	150	4	1. <u>Enterobacter cloacae</u>
3/15/78	2400	430	1. <u>Escherichia coli</u> 2. <u>Pseudomonas aeruginosa</u>
4/19/78	43	4	1. <u>Enterobacter agglomerans</u>
5/2/78	2400	29	N.D.
6/2/78	23	3	N.D.
6/26/78	2400	430	N.D.
7/25/78	24000	930	N.D.

* N.D. = Not Determined; Number preceding species name indicates order of predominance; i.e., "1" most predominant; "2" next most predominant; etc...

TABLE B-19. BACTERIAL FLORA OF LAKE FALLING CREEK (DEEP)
(SITE NO. 17)

Sampling Date	MPN	Fecal MPN	Predominant Gram Neg. Bacilli*
11/2/77	150	4	1. <u>Escherichia coli</u>
11/14/77	120	23	N.D.
12/12/77	4600	238	1. <u>Escherichia coli</u> 2. <u>Enterobacter cloacae</u>
2/6/78	230	3	N.D.
2/28/78	240	4	1. <u>Enterobacter cloacae</u>
3/13/78	4600	75	1. <u>Pseudomonas fluorescens</u> 2. <u>Enterobacter cloacae</u>
4/19/78	93	23	1. <u>Escherichia coli</u> 2. <u>Enterobacter cloacae</u>
5/2/78	16	16	N.D.
6/13/78	75	16	N.D.
6/26/78	4600	43	N.D.
7/25/78	240	15	N.D.

* N.D. = Not Determined; Number preceding species name indicates order of predominance; i.e., "1" most predominant; "2" next most predominant; etc...

TABLE B-20. BACTERIAL FLORA OF LAKE SWIFT CREEK
(SITE NO. 11)

Sampling Date	MPN	Fecal MPN	Predominant Gram Neg. Bacilli*
11/8/77	7500	23	N.D.
11/22/77	150	3	1. <u>Enterobacter cloacae</u>
12/6/77	230	3	1. <u>Enterobacter hafniae</u> 2. <u>Enterobacter cloacae</u>
12/20/77	43	3	N.D.
1/18/78	150	23	1. <u>Citrobacter freundii</u> 2. <u>Serratia marcescens</u>
1/31/78	2400	9	N.D.
3/7/78	1100	3	1. <u>Pseudomonas fluorescens</u>
4/12/78	3	15	N.D.
4/24/78	21	3	1. <u>Enterobacter cloacae</u>
5/11/78	4600	23	1. <u>Escherichia coli</u>
5/24/78	3	3	N.D.
7/10/78	15	3	N.D.
8/1/78	930	3	N.D.

* N.D. = Not Determined; Number preceding species name indicates order of predominance; i.e., "1" most predominant; "2" next most predominant; etc...

TABLE B-21. BACTERIAL FLORA OF LAKE SWIFT CREEK (DEEP)
(SITE NO. 12)

Sampling Date	MPN	Fecal MPN	Predominant Gram Neg. Bacilli*
11/8/77	1500	43	N.D.
11/22/77	7	4	1. <u>Escherichia coli</u>
12/6/77	36	3	N.D.
1/18/78	930	7	1. <u>Serratia marcescens</u> 2. <u>Klebsiella pneumoniae</u>
3/7/78	7	7	1. <u>Pseudomonas fluorescens</u>
4/12/78	3	7	N.D.
4/24/78	4	3	1. <u>Aeromonas hydrophilia</u>
5/11/78	430	9	1. <u>Escherichia coli</u>
5/24/78	40	3	N.D.
7/10/78	21	9	N.D.
8/1/78	430	4	N.D.

* N.D. = Not Determined; Number preceding species name indicates order of predominance; i.e., "1" most predominant; "2" next most predominant; etc...

TABLE B-22. WATER CHEMISTRY RESULTS - VPISU
(WATER COLLECTED MARCH 7 AND 8, 1977)

Lake	Alkalinity mg/l	Hardness mg/l	pH	Cond. μ mhos/cm	Temp. C°
Overhill	41	80	7.8	900	14
Salisbury	17	24	6.4	37	12
Pocahontas	10	24	6.7	48	12
Falling Creek	14	24	6.9	72	13
Manchester	27	56	7	110	14
Pallisades	16	36	7.2	58	14
Anna I	11	48	7.2	62	9
Anna II	15	48	7.2	58	10
Anna III	18	44	7	69	11

TABLE B-23. WATER CHEMISTRY RESULTS - VPISU
(WATER COLLECTED MARCH 7 AND 8, 1977)

Lake	Ortho PO ₄ -P mg/l	Total PO ₄ -P mg/l	NH ₃ -N mg/l	NO ₃ -N mg/l	NO ₂ -N mg/l	Org-N mg/l	SO ₄ mg/l	Cl mg/l
Overhill	<.025	<.025	<.1	.08	<.005	.00	76	147.5
Salisbury	<.025	.03	<.1		<.005	.62	9.3	75
Pocahontas	<.025	.05	<.1	.12	<.005	.08	10.5	125
Falling Creek	<.025	.04	.1	.19	<.005	.00	11	75
Manchester	<.025	.15	.29	.14	<.005	.20	23.6	225
Pallisades	<.025	.07	<.1	.06	<.005	.00	18.3	75
Anna I	<.025	.03	<.1	.15	<.005	.28	9.2	75
Anna II	<.025	.03	<.1	.16	<.005	.17	9.7	125
Anna III	<.025	.03	<.1	.13	<.005		9.5	75

TABLE B-24. WATER CHEMISTRY RESULTS - VPISU
(WATER COLLECTED MARCH 7 AND 8, 1977)

Lake	Mg mg/l	Mn mg/l	Pb mg/l	Zn mg/l	K mg/l	Na mg/l
Overhill	5.50	<.01	<.01	.80	5.5	206.0
Salisbury	.70	.06	.01	.17	2.0	4.6
Pocahontas	1.14	.18	<.01	.08	2.4	6.1
Falling Creek	1.69	.13	<.01	.42	2.5	7.9
Manchester	2.23	.09	.02	.01	4.6	7.1
Pallisades	.91	<.01	<.01	.03	3.4	4.6
Anna I	1.53	.17	<.01	.13	2.8	4.8
Anna II	1.48	.15	<.01	.03	2.8	4.9
Anna III	1.46	.13	.01	.07	3.0	4.9

TABLE B-25. WATER CHEMISTRY RESULTS - VPISU
(WATER COLLECTED MARCH 7 AND 8, 1977)

Lake	Cu mg/l	Ca mg/l	Cd mg/l	Cr mg/l	Fe mg/l
Overhill	.01	20.0	.02	<.01	.05
Salisbury	.01	2.46	.02	.01	.47
Pocahontas	<.01	3.13	.03	.14	.58
Falling Creek	<.01	4.00	<.01	<.01	.72
Manchester	.01	6.30	<.01	.02	1.19
Pallisades	.03	4.41	<.01	.01	.33
Anna I	.01	4.31	<.01	.02	.23
Anna II	.01	3.73	.01	.01	.28
Anna III	.01	3.87	.01	.02	.08

TABLE B-26. ORGANIC CARBON IN WATER SAMPLES*
COLLECTED MARCH 7 AND 8, 1977
(ANALYSES PERFORMED BY VPISU)

<u>Lake</u>	<u>Organic Carbon $\mu\text{g/ml}$</u>
Overhill	12.4
Salisbury	9.0
Pocahontas	6.6
Falling Creek	27.0
Manchester	19.6
Pallisades	5.9
Anna I	24.5
Anna II	29.3
Anna III	22.6

*Samples were frozen until April 18, 1977 when analyses were performed.

TABLE B-27. CHEMICAL CHARACTERIZATION OF SEDIMENT MATERIAL
OBTAINED ON MARCH 7 AND 8 THAT PASSED A #40
MESH SCREEN (ANALYSES BY VPISU)

Lake	Kjel.-N (mg/g)	O-PO ₄ -P (mg/g)	T-PO ₄ -P (mg/g)	Ash Free wt. (mg/g)
Overhill	--	0.056	0.070	3.5
Salisbury	0.307	0.092	0.080	34.8
Pochantas	0.240	0.027	0.030	8.1
Falling Creek	0.387	0.050	0.040	22.6
Manchester	0.316	0.203	0.130	47.0
Pallisades	0.299	0.094	0.090	22.0
Anna I	0.200	0.108	0.070	11.9
Anna II	0.179	0.144	0.240	31.7
Anna III	0.413	0.063	0.069	30.3

TABLE B-28. METALS FROM SEDIMENTS OBTAINED ON MARCH 7 AND 8
THAT PASSED A #40 MESH SCREEN
(ANALYSES BY VPISU)

Lake	Ca mg/g	Cu mg/g	Fe mg/g	Cd mg/g	Mg mg/g	Mn mg/g	Zn mg/g	Cr mg/g	K mg/g
Overhill	0.086	0.012	0.448	0.012	0.063	0.018	0.040	0.004	1.720
Salisbury	0.012	0.012	20.120	0.010	0.300	0.074	0.026	0.006	1.760
Pocohontas	0.036	0.008	2.160	0.010	0.046	0.046	0.022	0.042	1.660
Falling Creek	0.030	0.016	6.940	0.006	0.840	0.120	0.040	0.040	3.040
Manchester	0.046	0.030	24.400	0.008	1.580	0.148	0.044	0.066	5.260
Pallisades	0.016	0.152	7.900	0.012	0.082	0.030	0.036	0.048	2.660
Anna I	2.020	0.016	6.840	0.016	0.880	0.102	0.046	0.052	3.120
Anna II	0.092	0.030	24.960	0.012	24.440	1.040	0.048	0.062	3.840
Anna III	0.013	0.018	25.100	0.010	29.300	0.183	0.045	0.063	5.425

TABLE B-29. WATER CHEMISTRY RESULTS - VPISU
(WATER COLLECTED AUGUST 23, 1977)

Lake	Alk. mg/l	Hard. mg/l	pH	Cond. µmhos/cm	Temp. C
Moore's	90	24	7.90	1800	25.0
Pocahontas	26	36	6.96	50	25.0
Falling Creek	16	28	6.96	100	26.0
Manchester	15	32	6.85	35	27.3
Swift Creek	13	24	6.99	10	28.5
Salisbury	100	44	6.80	10	28.5
Overhill	40	72	6.73	2300	28.5
Anna I	20	36	7.20	100	30.0
Anna II	19	32	7.18	100	29.0
Anna III	18	32	7.19	50	29.0

TABLE B-30. WATER CHEMISTRY RESULTS - VPISU
(WATER COLLECTED AUGUST 23, 1977)

Lake	Ortho PO ₄ -P mg/l	Total PO ₄ -P mg/l	NH ₃ -N mg/l	NO ₃ -N mg/l	NO ₂ -N mg/l	Org-N mg/l	SO ₄ mg/l	Cl mg/l
Moore's	<0.025	0.11	0.48	0.060	<0.005	0.90	125.0	31,500.0
Pocahontas	<0.025	0.13	0.36	<0.005	<0.005	1.18	8.7	4.0
Falling Creek	0.030	0.16	0.36	0.060	0.008	1.46	14.3	54.0
Manchester	0.050	0.19	0.36	0.030	0.010	1.18	16.9	6.0
Swift Creek	<0.025	0.10	0.35	<0.005	<0.005	1.19	9.5	4.0
Salisbury	<0.025	0.18	0.36	<0.005	<0.005	3.10	11.7	4.0
Overhill	<0.025	0.15	0.39	0.080	<0.005	1.29	227.0	26,000.0
Anna I	<0.025	0.05	0.80	<0.005	<0.005	1.23	10.3	3.5
Anna II	<0.025	0.05	0.31	<0.005	<0.005	1.02	9.8	3.0
Anna III	<0.025	0.03	0.44	<0.005	<0.005	0.82	9.9	2.0

TABLE B-31. WATER CHEMISTRY RESULTS - VPISU
(WATER COLLECTED AUGUST 23, 1977)

Lake	Mg mg/l	Mn mg/l	Na mg/l	Zn mg/l	K mg/l
Moore's	15.0	0.01	160	0.20	3.0
Pocahontas	22.0	0.22	18	0.09	1.1
Falling Creek	19.0	0.13	13	0.77	2.0
Manchester	14.0	0.15	4	0.24	1.9
Swift Creek	21.0	0.04	9	0.47	1.0
Salisbury	29.0	0.26	28	0.07	1.0
Overhill	24.0	<0.01	162	0.04	5.0
Anna I	24.0	0.14	18	0.05	1.3
Anna II	23.0	0.09	17	0.06	1.3
Anna III	29.0	0.04	12	0.15	1.3

TABLE B-32. WATER CHEMISTRY RESULTS - VPISU
(WATER COLLECTED AUGUST 23, 1977)

Lake	Cu mg/l	Ca mg/l	Cd mg/l	Cr mg/l	Fe mg/l
Moore's	0.04	2.98	<0.01	0.10	1.90
Pocahontas	0.02	3.40	<0.01	<0.01	1.20
Falling Creek	0.04	6.60	<0.01	<0.01	1.50
Manchester	0.01	3.80	0.01	<0.01	2.50
Swift Creek	0.01	4.25	0.01	<0.01	0.30
Salisbury	0.01	2.28	<0.01	<0.01	0.50
Overhill	0.03	19.50	0.01	<0.01	0.60
Anna I	<0.01	3.88	<0.01	<0.01	0.50
Anna II	0.02	4.76	<0.01	<0.01	0.40
Anna III	0.01	4.88	<0.01	<0.01	0.40

TABLE B-33. ORGANIC CARBON IN WATER SAMPLES
COLLECTED ON AUGUST 23, 1977
(ANALYSES BY VPISU)

Lake	Organic Carbon mg/l
Moore's	1.0
Pocahontas	3.0
Falling Creek	4.9
Manchester	2.1
Swift Creek	3.1
Salisbury	9.0
Overhill	<1.0
Anna I	2.5
Anna II	2.1
Anna III	Lost

TABLE B-34. CHEMICAL CHARACTERIZATION OF SEDIMENT MATERIAL
OBTAINED ON AUGUST 23, 1977
(ANALYSES BY VPISU)

Lake	Kjel-N mg/g	Ortho PO ₄ -P mg/g	Total PO ₄ -P mg/g	Ash Free Wt. mg/g
Moore's	0.377	0.001	0.002	4.056
Pocahontas	0.356	0.001	0.002	5.430
Falling Creek	2.237	0.004	0.002	82.226
Manchester	0.497	0.003	0.003	12.831
Swift Creek	0.618	0.002	0.002	23.918
Salisbury	2.437	0.004	0.004	79.747
Overhill	0.242	0.002	0.002	3.902
Anna I	0.301	0.002	0.002	6.411
Anna II	0.431	0.003	0.002	8.612
Anna III	0.607	0.004	0.004	44.454

TABLE B-35. METALS FROM SEDIMENTS OBTAINED ON AUGUST 23, 1977
(ANALYSES BY VPISU)

Lake	K mg/g	Na mg/g	Cd mg/g	Cr mg/g
Moore's	0.038	0.210	<0.010	0.019
Pocahontas	0.010	0.039	<0.010	0.019
Falling Creek	0.957	0.077	<0.010	0.029
Manchester	0.825	0.132	<0.010	0.016
Swift Creek	0.102	0.017	<0.010	<0.010
Salisbury	0.419	0.057	<0.010	0.019
Overhill	0.036	0.091	<0.010	<0.010
Anna I	0.732	0.055	<0.010	0.018
Anna II	2.368	0.079	<0.010	<0.010
Anna III	4.182	0.080	<0.010	0.040

TABLE B-36. METALS FROM SEDIMENTS OBTAINED ON AUGUST 23, 1977
(ANALYSES BY VPISU)

Lake	Ca mg/g	Cu mg/g	Fe mg/g	Mg mg/g	Mn mg/g	Zn mg/g
Moore's	0.038	0.002	4.008	0.401	0.019	0.057
Pocahontas	<0.010	0.008	4.062	0.416	0.077	<0.010
Falling Creek	0.019	0.136	19.340	10.914	0.191	0.057
Manchester	<0.019	0.053	10.393	8.248	0.082	0.016
Swift Creek	<0.019	0.022	9.328	4.919	0.848	<0.010
Salisbury	<0.019	0.149	24.941	6.092	0.095	0.038
Overhill	<0.010	0.005	2.733	0.346	0.018	<0.010
Anna I	<0.010	0.459	5.858	10.618	0.164	0.018
Anna II	<0.010	0.160	8.682	26.046	0.256	0.020
Anna III	0.020	0.217	24.096	43.015	0.438	0.040

TABLE B-37. NUMBERS OF PROTOZOAN SPECIES BY ORDERS (FLAGELLATES AND SARCODINES) AND SUBCLASSES (CILIATES) IDENTIFIED IN PLANKTON SAMPLES FROM RICHMOND, VIRGINIA, AREA LAKES WATER SAMPLES OBTAINED MARCH 7 AND 8, 1977 (ANALYSES BY VPISU)

Protozoans	Lakes								
	Overhill	Salisbury	Pocahontas	Falling Creek	Manchester	Pallisades	Anna I	Anna II	Anna III
Chrysomonads	1	3	8	6	2	2	8	7	6
Cryptomonads				2		1	2	2	2
Phytomonads	2		3	1		1			
*Euglenoids-Chloro			4	3	4	1	5	7	5
†Euglenoids-C-less	1		3					1	
Dinoflagellates	2	2	1	2		2	1	3	3
Chloromonads				1					
Zoomastigophora	1								
Proteomyxida									
Amoebida							1	2	
Testaceans	2	4	1		4	2		1	
Heliozoans	1						1	1	1
Holotrichs	1	1			2	1	1	1	3
Spirotrichs		2	1	1	1	1	1	1	
Peritrichs	1					1	1	1	2
Totals	12	12	21	16	13	12	21	27	22

* Euglenoids with chromatophores.

† Euglenoids that are colorless.

TABLE B-38. NUMBERS OF GENERA OF NON-PROTOZOAN MICROBIOTA IDENTIFIED IN PLANKTON SAMPLES FROM RICHMOND, VIRGINIA, AREA LAKES (WATER SAMPLES OBTAINED MARCH 7, AND 8, 1977. ANALYSIS BY VPISU).

Microbiota	Lakes								
	Overhill	Salisbury	Pocahontas	Falling Creek	Manchester	Palisades	Anna I	Anna II	Anna III
Cyanophytes		1	1	1		1	3	2	2
Desmids	1	8	4	2	4	3	3		
Other Green Algae	1	3	1	1		1			
Diatoms		2	7	5	4	1	7	7	9
Sulfur Bacteria			1	1					
Rotifers		2	3	4	2	3	1	3	2
Gastrotrichs					1				
Chaetogaster					1				
Nematodes			1						
Insects	2								
Nauplii		1		1	1	1		1	
Copepods		1	1		1		1	1	1
Ostracods									
Cladocerans		3	1	1	2			2	1
Totals	4	21	20	16	16	10	16	16	15

TABLE B-39. NUMBERS OF PROTOZOAN SPECIES BY ORDERS (FLAGELLATES AND SARCODINES)
AND SUBCLASSES (CILIATES) IDENTIFIED FROM
POLYURETHANE FOAM UNITS AFTER 14 DAYS EXPOSURE TO SOME
RICHMOND, VIRGINIA, AREA LAKES
SAMPLES OBTAINED MARCH, 1977
(ANALYSES BY VPISU)

Protozoans	Lakes				
	Overhill	Salisbury	Pocahontas	Falling Creek	Manchester
Chrysomonads	4	2	1	3	5
Cryptomonads		3	2	3	3
Phytomonads					
*Euglenoids-Chloro	2	1	5	1	2
+Euglenoids-C-less	4		5	3	6
Dinoflagellates		1	1		1
Chloromonads					
Zoomastigophora	2		2	3	3
Proteomyxida				1	
Amoebida	2		2	2	4
Testaceans	1	1	3	2	2
Heliozoans	1		1	1	2
Holotrichs	4	9	15	7	9
Spirotrichs	2	8	6	4	7
Peritrichs	1	2		1	
Totals	23	27	43	31	44

* Euglenoids with chromatophores.

+ Euglenoids that are colorless.

TABLE B-40. NUMBERS OF GENERA OF NON-PROTOZOAN MICROBIOTA IDENTIFIED
FROM POLYURETHANE FOAM UNITS AFTER 14 DAYS EXPOSURE TO
SOME RICHMOND, VIRGINIA, AREA LAKES
SAMPLES OBTAINED IN MARCH, 1977
(ANALYSES BY VPISU)

Microbiota	Lakes				
	Overhill	Salisbury	Pocahontas	Falling Creek	Manchester
					Anna I
Cyanophytes	2	1	1	2	3
Desmids	1	4	6	4	5
Other Green Algae	1	5	1	1	2
Diatoms	2	5	11	10	4
Sulfur Bacteria	1				11
Rotifers	2	5	6	2	1
Gastrotrichs		1	1	1	4
Chaetogasters		1			1
Nematodes		1	1	1	1
Insects	1				
Nauplii	1	1	1	1	
Copepods	2				
Ostracods		1	1	2	1
Cladocerans		1			
Totals	13	26	29	24	17
					26

TABLE B-41. RANKING OF SOME RICHMOND, VIRGINIA, LAKES ACCORDING TO
TOTAL NUMBERS OF PLANKTONIC PROTOZOANS
(ANALYSES OF VPISU)

Rank	Lake	Number of Protozoans	Organic Carbon	Plankton Biota		
				Desmids	Diatoms	Rotifers
1	Anna II	27	29.3	0	7	3
2	Anna III	22	22.6	0	9	2
3	Anna I	21	24.5	3	7	1
4	Pocahontas	21	6.6	4	7	3
5	Falling Creek	16	27.0	2	1	4
6	Manchester	13	19.6	4	4	2
7	Overhill	12	12.4	1	0	0
8	Salisbury	12	9.0	8	2	2
9	Palisades	12	5.9	3	1	3